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(54) Title: CD28 SYNTHEBODY FOR THE MODULATION OF IMMUNE RESPONSES

(57) Abstract: The present invention provides variants of an immunoglobulin variable domain having at least one CDR region and framework regions flanking the CDR. The variant includes (a) the CDR region having added or substituted therein at least one binding sequence and (b) the flanking framework regions, wherein the binding sequence is heterologous to the CDR and is a binding sequence from a binding site of a binding pair, and wherein the binding sequence is a CD80 and/or CD86 receptor-binding portion of CD28. Also disclosed and claimed are molecules, immunoglobulins, and compositions including these variants, as well as nucleic acids and expression vectors encoding these variants, and methods of using the variants for immunotherapy.

CD28 SYNTHEBODY FOR THE MODULATION OF IMMUNE RESPONSES

FIELD OF THE INVENTION

The present invention relates to the modulation of immunological disorders. In particular, the invention provides a synthetic antibody-based approach to inhibit T lymphocyte activation. The synthetic antibodies are useful in the treatment of autoimmune diseases, allergic responses, transplant rejection and other diseases associated with excessive or inappropriate activity of T lymphocytes.

BACKGROUND OF THE INVENTION

Antigens activate T lymphocytes using a two-signal pathway. Specificity is provided by engagement of the T lymphocyte receptor with a complex composed of a peptide derived from the antigen and the MHC molecule on antigen-presenting cells. This interaction alone is necessary but insufficient to induce optimal T lymphocyte activation, but can be significantly enhanced with costimulatory signals. The strongest costimulatory signal for the interaction between T lymphocytes and antigen-presenting cells is provided by CD28 on T lymphocytes binding to its receptors, CD80 and CD86, on antigen-presenting cells. Costimulation through CD28 results in enhanced activation, proliferation and differentiation of T lymphocytes (J.A. Bluestone, 1995, Immunity 2:555-559).

Autoimmune disease encompasses more than 80 chronic disabling diseases and disorders that target almost every organ in the body. Over 50 million people suffer from one or more autoimmune diseases. A number of strategies have been proposed to suppress autoimmune disease. These strategies include use of immunosuppressive drugs such as cyclophosphamide, cyclosporin A, methotrexate and Imuran (azathioprine); steroids such as prednisone and methylprednisolone ; and immunosuppressive cytokines such as IL-10. The drawbacks of these drugs are that their long-term efficacy against cell and antibody-mediated immune diseases is limited. Further drawbacks include severe side effects and general immunosuppression. One potential consequence of prolonged immunosuppression is an increased risk of hematologic malignancy.

Inappropriate CD28 binding to CD80 has been shown to play a key role during the course of autoimmune disease (Daikh *et al.*, 1997, J. Leukoc. Biol., 62:156-162

and Nakajima and Azuma, 1997, Nippon Rinsho, 55:1419-1424). It has also been shown that disruption of the interaction of CD28 with CD80 and CD86 alters the immune response to self antigens and prevents the development of autoimmunity in mice. CD28 overexpression may play a role in the clinical progression of multiple myeloma (Robillard *et al.*, 1998, Clin. Cancer Res., 4:1521-1526). CD28 also contributes to the pathogenesis and the severity of graft-vs-host-disease (GVHD) and the severity of GVHD can be decreased by the administration of agents that block CD28 function in lymphocytes (Yu *et al.*, 1998, Blood, 92:2963-2970). In a recent study, CD28 binding to its co-receptors and T lymphocyte activation were shown to play important roles in the pathogenesis of Wegener's granulomatosis (Moosig *et al.*, 1998, Clin. Exp. Imunol., 114:113-118). Patients with Wegener's granulomatosis show a reduced expression of CD28 on T lymphocytes and an up-regulation of CD80 and CD86 on T lymphocytes after activation. This up-regulation of CD80 and CD86 may contribute to the persistently high levels of immune activation observed in patients with Wegener's granulomatosis.

Antibodies specific for CD28 have been shown to block the binding of CD28 to CD80 and CD86 and have been used to investigate the importance of different domains of CD28 in the binding interaction (Parry *et al.*, *supra* and Truneh *et al.*, *supra*). Bispecific antibodies specific for tumor antigen and CD28 have been shown to provide an effective approach to tumor vaccines by augmenting the activation of tumor antigen specific effector T lymphocytes bearing the CD28 receptor (Demanet *et al.*, 1996, Blood, 87:4390-4398; Hayden *et al.*, 1996, Tissue Antigens, 48:242-254; and, Guo *et al.*, 1997, Nat. Med., 3:451-455).

CD28

CD28 is a T lymphocyte-specific transmembrane glycoprotein that is involved in T lymphocyte activation (Linsley and Ledbetter, 1993, Annu. Rev. Immunol., 11:191-212). It is a type I membrane homodimer that contains a single V-like domain and belongs to the immunoglobulin superfamily. The unprocessed precursor consists of 220 amino acids and has a molecular weight of approximately 25 kDa (Aruffo and Seed, 1987, Proc. Nat'l. Acad. Sci. USA, 84:8573-8577). CD28 shows strong homology with CTLA-4, found on the surface of cytotoxic T lymphocytes and activated CD4+ T lymphocytes. Activation of naïve CD4+ T lymphocytes requires two discrete signals: a signal delivered by the T lymphocyte receptor following recognition of antigen and an accessory signal produced when co-stimulatory receptors interact with their ligands. CD28 present on the surface of T lymphocytes produces

a potent co-stimulatory signal when engaged by the co-receptors CD80 and CD86 on antigen presenting cells and T lymphocyte activation can be inhibited by blocking CD28 binding to CD80/86 (Damle, *et al*, 1981, Proc. Natl. Acad. Sci. USA, 78:5096-5098 and Damle, *et al*, 1988, J. Immunol., 140:1753-1761).

The intracellular signaling pathways activated upon binding of CD28 to CD80 and CD86 involves activation of phosphatidylinositol (PI)3-kinase that subsequently results in the activation of Akt/protein kinase B (PKB) (Arrufo and Seed, *supra*). PKB plays a major role in the regulation of cell survival and the activation of PKB may result in the protective effects of CD28 seen in different cell and animal models (Parry *et al.*, 1998, Eur. J. Imunol., 27:2495-2501). The CD28 signal also acts in concert with the T lymphocyte receptor signal to activate the NF-kappaB family of transcription factors, which play a role in the regulation of the interleukin-2 gene (Harhaj and Sun, 1998, J. Biol. Chem., 273:25185-25190).

Using site directed mutagenesis of CD28, the peptide sequence "MYPPPY" (SEQ ID NO: 1) was identified in the CDR3 region of the V-like domain as a key site for binding of CD28 to its binding partners (or receptors) CD80 and CD86 (Kariv *et al.*, 1996, J. Immunol., 157:29-38 and Truneh *et al.*, 1996, Mol. Immunol., 33:321-334). Mutational analysis has also been used to identify the amino acid sequence of CD80 that interacts with CD28 (Guo *et al.*, 1995, J. Exp. Med., 181:1345-1355).

Immunoglobulins and Immune Response

The basic unit of antibody immunoglobulin structure is a complex of four polypeptides -- two identical low molecular weight or "light" chains and two identical high molecular weight or "heavy" chains -- linked together by both non-covalent associations and by disulfide bonds. Each light and heavy chain of an antibody has a variable region at its amino terminus and a constant domain at its carboxyl terminus. The variable regions are distinct for each antibody and contain the antigen binding site. Each variable domain is comprised of four relatively conserved framework regions and three regions of sequence hypervariability termed complementarity determining regions or "CDRs". For the most part, it is the CDRs that form the antigen binding site and confer antigen specificity. The constant domains are more highly conserved than the variable regions, with slight variations due to haplotypic differences.

Based on their amino acid sequences, light chains are classified as either kappa or lambda. The constant region of heavy chains is composed of multiple domains

(CH1, CH2, CH3. . . CHx), the number depending upon the particular antibody class. The CH1 region is separated from the CH2 region by a hinge region that allows flexibility in the antibody. The variable region of each light chain aligns with the variable region of each heavy chain, and the constant region of each light chain aligns with the first constant region of each heavy chain. The CH2-CHx domains of the constant region of a heavy chain form an "Fc region" that is responsible for the effector functions of the immunoglobulin molecule, such as complement binding and binding to the Fc receptors expressed by lymphocytes, granulocytes, monocyte lineage cells, killer cells, mast cells, and other immune effector cells.

In modern medicine, immunotherapy or vaccination has virtually eradicated diseases such as polio, tetanus, tuberculosis, chicken pox, measles, hepatitis, etc. The approach using vaccinations has exploited the ability of the immune system to prevent infectious diseases.

There are two types of immunotherapy, active immunotherapy and passive immunotherapy. In active immunotherapy, an antigen is administered in a vaccine to a patient so as to elicit a long-lasting protective immune response against the antigen. Passive immunotherapy involves the administration of protective antibodies to a patient to elicit an acute immune response that lasts only as long as the antibody is present. Antibody therapy is conventionally characterized as passive since the patient is not the source of the antibodies. However, the term passive may be misleading because a patient may produce antiidiotype secondary antibodies, which in turn provoke an immune response that is cross-reactive with the original antigen. Immunotherapy where the patient generates antiidiotypic antibodies is often more therapeutically effective than passive immunotherapy because the patient's own immune system generates an active immune response against cells bearing the particular antigen well after the initial infusion of protective antibody has cleared from the system.

PCT Publication WO 99/25378 relates to synthebody molecules, particularly antibodies, that bind one member of a binding pair and have at least one complementarity determining region (CDR) that contains the amino acid sequence of a binding site for that member of the binding pair. The binding site is derived from the other member of the binding pair. It also relates to methods for treating, diagnosing, or screening for diseases and disorders associated with the expression of the member of the binding pair using the modified antibodies.

PCT Publication WO 99/25379 relates to vaccine compositions of antibodies in which one or more variable region cysteine residues that form intrachain disulfide bonds have been replaced with amino acid residues that do not contain a sulfhydryl group and,

therefore, do not form disulfide bonds. It also relates to use of the vaccine compositions to treat or prevent certain diseases and disorders.

In sum, there is a need in the art to identify effective methods to target the source of the immune dysfunction characteristic of autoimmune diseases. There is a further need in the art to inhibit over proliferation of T lymphocytes associated with autoimmune diseases and the other conditions mentioned above. The present invention addresses these and other needs in the art with the discovery of an effective antibody-based CD28 antagonist system.

SUMMARY OF THE INVENTION

The present invention provides variants of an immunoglobulin variable domain. The immunoglobulin variable domain comprises (A) at least one CDR region and (B) framework regions flanking said CDR. The variant comprises (a) the CDR region having added or substituted therein at least one binding sequence and (b) the flanking framework regions, wherein the binding sequence is heterologous to the CDR and is a binding sequence from a binding site of a binding pair, and wherein said binding sequence is a CD80 and/or CD86 receptor-binding portion of CD28.

In further embodiment, (i) one or more amino acid residues in one or more of the flanking framework regions has been substituted or deleted, (ii) one or more amino acid residues has been added in one or more of the flanking framework regions, or (iii) a combination of (i) and (ii). Alternatively, (i) one or more amino acid residues in one or more framework regions other than the framework regions flanking the CDR has been substituted or deleted, (ii) one or more amino acid residues has been added in one or more framework regions other than the framework regions flanking said CDR, or (iii) a combination of (i) and (ii). In yet another alternative, (i) one or more amino acid residues in one or more of the flanking framework regions has been substituted or deleted, (ii) one or more amino acid residues has been added in one or more of the flanking framework regions, or (iii) a combination of (i) and (ii); and (iv) one or more amino acid residues in one or more framework regions other than the framework regions flanking the CDR has been substituted or deleted, (v) one or more amino acid residues has been added in one or more framework regions other than the framework regions flanking said CDR, or (vi) a combination of (iv) and (v).

The present invention also provide variants in which the CDR region has added or substituted therein at least one amino acid sequence which is heterologous to the

CDR and the flanking framework regions, wherein the heterologous sequence is capable of binding to a target sequence or molecule, and wherein the heterologous sequence is a CD80 and/or CD86 receptor-binding portion of CD28. Again, (i) one or more amino acid residues in one or more of the flanking framework regions may be substituted or deleted, (ii) one or more amino acid residues may be added in one or more of the flanking framework regions, or (iii) a combination of (i) and (ii); (i) one or more amino acid residues in one or more framework regions other than the framework regions flanking the CDR may be substituted or deleted, (ii) one or more amino acid residues may be added in one or more framework regions other than the framework regions flanking the CDR, or (iii) a combination of (i) and (ii), or (i) one or more amino acid residues in one or more of the flanking framework regions may be substituted or deleted, (ii) one or more amino acid residues may be added in one or more of the flanking framework regions, (iii) a combination of (i) and (ii); and (iv) one or more amino acid residues in one or more framework regions other than the framework regions flanking the CDR may be substituted or deleted, (v) one or more amino acid residues may be added in one or more framework regions other than the framework regions flanking the CDR, or (vi) a combination of (iv) and (v).

The invention also provides molecules comprising the variants described herein. The molecules can include one or more constant domains from an immunoglobulin; a second variable domain associated with the variant such as, for example, a variable domain of a heavy chain is associated with a variable domain of a light chain in an immunoglobulin; and a second variable domain associated with the variant, with one or more constant domains from immunoglobulins.

Also provided are immunoglobulins comprising a heavy chain and a light chain, wherein said heavy chain comprises a variant as described above and three constant domains from an immunoglobulin heavy chain, and the light chain comprises a second variable domain associated with the variant and a constant domain from an immunoglobulin light chain. Furthermore, the present invention provides immunoglobulins comprising a heavy chain and a light chain, wherein the light chain comprises a variant as described above and a constant domain from an immunoglobulin light chain, and the heavy chain comprises a second variable domain associated with said variant and three constant domains from an immunoglobulin heavy chain.

Isolated nucleic acids encoding these variants, molecules, and immunoglobulins are also provided, as are cells containing these nucleic acids. Recombinant non-human hosts containing these nucleic acids are also provided. Pharmaceutical

compositions comprising a therapeutically or prophylactically effective amount of the variants, molecules or immunoglobulins and pharmaceutically acceptable carriers are also provided.

The invention further provides pharmaceutical compositions that comprise an amount of the synthetic antibody effective to bind CD80 and CD86. These compositions may further include a pharmaceutically acceptable carrier or excipient.

Preferably, the synthetic antibody comprises one or more sequences MYPPPY (SEQ ID NO:1), more preferably EVMYPPPYLDN (SEQ ID NO:2), preferably in CDR3 of a human light chain variable region. A pharmaceutical composition or vaccine composition, as set forth above, comprises this synthetic antibody.

In a further embodiment, the invention provides a nucleic acid encoding the synthetic antibody. The invention also furnishes pharmaceutical compositions comprising the nucleic acid encoding the synthetic antibody in an amount effective to produce sufficient amounts of the antibody to bind CD80+ and/or CD86+ (i.e., the CD80/86 co-receptor). These compositions may further include a pharmaceutically acceptable carrier or excipient.

Also encompassed are expression vectors, in which the nucleic acid is operably associated with an expression control sequence. The invention extends to host cells transfected or transformed with the expression vector. The synthetic antibody or nucleic acid can be produced by isolating it from the host cells grown under conditions that permit production of the nucleic acid or expression of the synthetic antibody.

The pharmaceutical and vaccine compositions of the invention can be administered to a subject to modulate CD28-mediated lymphocyte activity, and particularly to inhibit immune activation associated with CD28-mediated binding.

DESCRIPTION OF THE DRAWINGS

Figure 1A and 1B. Consensus amino acid sequences of (A) the heavy chain variable region (SEQ ID NO:3) and (B) the light chain variable region (SEQ ID NO: 4).

Figure 2. Diagram of PCR kitting strategy.

Figure 3. Binding of CD28 antibody variant to Raji B lymphoma cells evaluated by flow cytometry.

DETAILED DESCRIPTION

The present invention provides an approach for the modulation of immune responses in mammals. In one aspect, a synthetic antibody competitively inhibits the interaction of CD28 with the ligands CD86 and CD80, thus inhibiting immune activation. Inhibition of immune activation is beneficial for the treatment of autoimmune diseases, allergy, transplant rejection, and other T lymphocyte mediated immune dysfunctions.

Thus, in one aspect, the invention provides a synthetic antibody (synthebody) that contains a binding site for CD86 and CD80 in a CDR, which CDR is flanked by framework regions of a variable region. The invention further provides pharmaceutical compositions that comprise an amount of the synthebody effective to inhibit binding of CD28 to its co-receptors CD80 and CD86 *in vivo* and a pharmaceutically acceptable carrier or excipient.

Recombinant nucleic acids, particularly DNA molecules, provide for efficient expression of the foregoing synthebodies. In one aspect of this embodiment, the invention provides a nucleic acid encoding the synthebody. Also encompassed are expression vectors in which the nucleic acid is operably associated with an expression control sequence. The invention extends to host cells transfected or transformed with the expression vector. The synthebody can be produced by isolating it from the host cells grown under conditions that permit expression of the synthebody.

The invention also furnishes a pharmaceutical composition comprising the vector that expresses the synthebody in an amount effective to produce sufficient synthebody to inhibit binding of CD28 to its co-receptors CD80 and CD86 *in vivo*, and a pharmaceutically acceptable carrier or excipient.

The pharmaceutical and vaccine compositions of the invention can be administered to a subject with diseases involving dysfunction of immune system modulation, including arthritis, asthma, type I (autoimmune) diabetes mellitus, multiple sclerosis, multiple myeloma, and systemic lupus erythematosis. Such conditions affect more than 1 in 5 people (approximately 52 million) in the United States (Sources: National Institute of Health and Center for Disease Control). The major clinical indications for CD28 synthebody are the treatment of autoimmune diseases and in the prevention of graft versus host disease.

Alternatively, the constructs of the invention can be used to prolong an established cytoxic T lymphocyte-dependent immune response by inhibiting the down-regulatory effects of CTLA4 activation by binding CD80 and/or CD86. Both CTLA4 and CD28 share the same CD80 and CD86-specific binding sequence MYPPPY (SEQ ID NO: 1).

While CD28 binding to CD80 and/or CD86 activates T lymphocytes, CTLA4 binding suppresses or modulates T lymphocyte activation, particularly cytotoxic T lymphocyte activation. Thus, while administering a CD80/CD86-binding construct of the invention suppresses immune activation, e.g., at inception of an immune response, the same construct can enhance or prolong an established immune response. This can be especially valuable for enhancing amnestic responses to pathogens against which a subject has been vaccinated; for enhancing immunotherapy of infections; and for improving anti-tumor cytotoxicity.

Because of their specific binding to CD80 and CD86, constructs of the invention can be used to detect either of these molecules, e.g., by FACS analysis (as shown in the Examples).

The present invention is based, in part, on the development of a synthetic antibody by insertion of a receptor-binding portion of CD28 (preferably corresponding to residues 117-122 of the amino acid sequence of CD28) into CDR3 of a consensus light chain variable region. This synthebody construct binds to the co-receptors CD80/86 with high affinity. In addition, it blocks the interaction between cells expressing CD28 and cells expressing CD80 and/or CD86 to inhibit T lymphocyte activation.

The term "immune activation" as used herein refers to activation of T lymphocytes and the sequelae of such T lymphocyte activation, particularly in connection with cellular and humoral immunity. More particularly, the invention relates to inhibition of immune activation mediated by CD28 binding to CD80/86 co-receptors, as discussed above, where such immune activation results in or is associated with an undesirable immune response or immune dysfunction. An undesirable immune response includes, without limitation, acute and chronic transplant rejection (host-versus graft disease), graft-versus-host disease, and pathological inflammatory conditions, e.g., associated with contact sensitivity, allergy, or excessive response to an infection. Undesirable immune response (or immune dysfunction) specifically includes any autoimmune disease.

An "autoimmune disease" is a malfunction of the immune system, i.e., a pathological condition in which the immune system of a mammal (including a human) ceases or fails to recognize self (i.e., autologous antigens) and as a result treats these self antigens as if they were foreign antigens and mounts an immune response against them. Non-limiting examples of autoimmune disease include multiple sclerosis, Type 1 diabetes, rheumatoid arthritis, lupus erythematosis, and autoimmune thyroididites.

The term "construct" refers to the variant of a variable domain of an immunoglobulin superfamily protein, including molecules comprising such variants,

described herein. The immunoglobulin superfamily is well known, and includes antibody/B-cell receptor proteins, T lymphocyte receptor proteins, and other proteins mentioned *infra* (*see*, Paul, Fundamental Immunology, 3rd Ed.). The modification refers to insertion into or substitution of a portion of the immunoglobulin superfamily protein sequence with a heterologous amino acid sequence or heterologous binding sequence. The site of substitution in the immunoglobulin superfamily protein corresponds to a binding-accessible portion of the region of the immunoglobulin superfamily protein, *e.g.*, a region that corresponds to an antibody variable region, and more particularly a portion corresponding to a CDR of an antibody variable region.

A "synthebody" (for synthetic antibody) is a specific example of a construct of the invention that includes an antibody variable region. It may also include regions corresponding to an antibody constant region or regions, or be associated with one or more other immunoglobulin family polypeptides, such as an antibody Fv heterodimer, an antibody tetramer, a T lymphocyte receptor heterodimer, etc. Embodiments described below are illustrative of the variants and molecules of the present invention in that the variants are included in synthebodies and synthebodies are a type of molecule that includes the variants. The term "synthebody" thus refers to an illustrative example of a type of construct of the invention.

The term "heterologous" refers to a combination of elements not naturally occurring in a particular locus. For example, heterologous DNA refers to DNA not naturally located in the cell or in a particular chromosomal site of the cell. A heterologous expression regulatory element is such an element operatively associated with a different gene than the one it is operatively associated with in nature. In the context of the present invention, a construct coding sequence is heterologous to the vector DNA in which it is inserted for cloning or expression and it is heterologous to a host cell containing such a vector in which it is expressed, *e.g.*, a CHO cell. Moreover, the constructs of the present invention contain a heterologous DNA, amino acid, or binding sequence.

The "heterologous amino acid (or binding) sequence" (also "binding sequence") refers to the desired binding segment of a polypeptide, *e.g.*, the portion of a polypeptide (protein or peptide) that binds to a receptor. As used in this application, the term refers to the sequence of the CD80 and/or CD86 binding site of CD28.

A "target receptor" or "target binding partner" (also simply "target") is a molecule that is recognized by and specifically bound by the molecule from which the

heterologous binding sequence is derived (CD28), and which is therefore, recognized and specifically bound by a construct. In particular, the target receptor is CD80 or CD86, or both.

The term "CDR" refers to a part of the variable region of an immunoglobulin family protein that confers binding specificity, *e.g.*, antibody specificity for antigen. In antibodies, CDRs are highly variable and accessible. The site of introduction of the heterologous CD80 and/or CD86 receptor binding sequence from CD28 is termed herein a "CDR".

The term "framework region" refers to the part of the modified immunoglobulin molecule corresponding to an antibody framework region, as defined in the art. Sequences flanking the CDR are termed herein "framework regions of a variable region".

The term "flanked" and "flanking" refers to the amino acids that are connected to or are connected by spacing amino acids to the protein sequence of the CDR. "Spacing amino acids" (or a "spacer group") are amino acids that are not found in the native framework sequence or the CDR or the substituted sequence, nor do they independently confer any binding activity on the modified variable region. They may be included to preserve or ensure a proper variable region conformation and orientation of the CDR or substituted heterologous amino acid sequence.

The phrase "pharmaceutically acceptable", whether used in connection with the pharmaceutical compositions of the invention or vaccine compositions of the invention, refers to molecular entities and compositions that are physiologically tolerable and do not typically produce untoward reactions when administered to a human. Preferably, as used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or aqueous solution saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin, 18th Edition.

The term "about" or "approximately" will be known to those skilled in the art in light of this disclosure. Preferably, the term means within 20%, more preferably within 10%, and more preferably still within 5% of a given value or range. Alternatively, especially

in biological systems, the term "about" preferably means within about a log (*i.e.*, an order of magnitude) preferably within a factor of two of a given value, depending on how quantitative the measurement.

5

Molecular Biology - Definitions

A "coding sequence" or a sequence "encoding" an expression product, such as a RNA, polypeptide, protein, or enzyme, is a nucleotide sequence that, when expressed, results in the production of that RNA, polypeptide, protein, or enzyme, *i.e.,* the nucleotide
10 sequence encodes an amino acid sequence for that polypeptide, protein or enzyme. A coding sequence for a protein may include a start codon (usually ATG) and a stop codon.

The term "gene", also called a "structural gene" means a DNA sequence that codes for or corresponds to a particular sequence of amino acids which comprise all or part of one or more proteins, and may or may not include regulatory DNA sequences, such as
15 promoter sequences, that determine for example the conditions under which the gene is expressed. The transcribed region of a gene can include 5'- and 3'-untranslated regions (UTRs) and introns in addition to the translated (coding) region.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding
20 sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined for example, by mapping with nuclease S1), as well as
25 protein binding domains (consensus sequences) responsible for the binding of RNA polymerase.

A coding sequence is "under the control" of or "operably associated with" transcriptional and translational control sequences in a cell. RNA polymerase transcribes the coding sequence into mRNA, which is then trans-RNA spliced (if it contains introns) and
30 translated into the protein encoded by the coding sequence.

The terms "express" and "expression" mean allowing or causing the information in a gene or DNA sequence to become manifest, for example producing a protein by activating the cellular functions involved in transcription and translation of a corresponding gene or DNA sequence. A DNA sequence is expressed in or by a cell to form

an "expression product" such as a mRNA or a protein. The expression product itself, *e.g.* the resulting mRNA or protein, may also be said to be "expressed" by the cell. An expression product can be characterized as intracellular, extracellular or secreted. The term "intracellular" means something that is inside a cell. The term "extracellular" means something that is outside a cell. A substance is "secreted" by a cell if it appears in significant measure outside the cell, from somewhere on or inside the cell. "Conditions that permit expression" *in vitro* are culture conditions of temperature (generally about 37^0C), humidity (humid atmosphere), carbon dioxide concentration to maintain pH (generally about 5% CO_2 to about 15% CO_2), pH (generally about 7.0 to 8.0, preferably 7.5), and culture fluid components that depend on host cell type. *In vivo*, the conditions that permit expression are primarily the health of the non-human transgenic animal, which depends on adequate nutrition, water, habitation, and environmental conditions (light-dark cycle, temperature, humidity, noise level). In either system, expression may depend on a repressor or inducer control system, as well known in the art.

The term "transfection" means the introduction of a "foreign" (*i.e.,* extrinsic or extracellular) gene, DNA or RNA sequence into a host cell, so that the host cell will express the introduced gene or sequence to produce a desired substance, typically a protein or enzyme encoded by the introduced gene or sequence. The introduced gene or sequence may also be called a "cloned" or "foreign" gene or sequence, may include regulatory or control sequences, such as start, stop, promoter, signal, secretion, or other sequences used by a cell's genetic machinery. The gene or sequence may include nonfunctional sequences or sequences with no known function. A host cell that receives and expresses introduced DNA or RNA has been "transfected" and is a "transfectant" or a "clone." The DNA or RNA introduced to a host cell can come from any source, including cells of the same genus or species as the host cell, or cells of a different genus or species.

The terms "vector", "cloning vector" and "expression vector" mean the vehicle by which a DNA or RNA sequence (*e.g.* a foreign gene) can be introduced into a host cell, so as to transform the host and promote expression (*e.g.* transcription and translation) of the introduced sequence. Vectors include plasmids, phages, viruses, etc.; they are discussed in greater detail below.

Vectors typically comprise the DNA of a transmissible agent, into which foreign DNA is inserted. A common way to insert one segment of DNA into another segment of DNA involves the use of enzymes called restriction enzymes that cleave DNA at specific sites (specific groups of nucleotides) called restriction sites. A "cassette" refers to a

DNA segment that can be inserted into a vector or into another piece of DNA at a defined restriction site. Preferably, a cassette is an "expression cassette" in which the DNA is a coding sequence or segment of DNA that codes for an expression product that can be inserted into a vector at defined restriction sites. The cassette restriction sites generally are designed to ensure insertion of the cassette in the proper reading frame. Generally, foreign DNA is inserted at one or more restriction sites of the vector DNA, and then is carried by the vector into a host cell along with the transmissible vector DNA. A segment or sequence of DNA having inserted or added DNA, such as an expression vector, can also be called a "DNA construct." A common type of vector is a "plasmid" that generally is a self-contained molecule of double-stranded DNA, usually of bacterial origin, that can readily accept additional (foreign) DNA and which can be readily introduced into a suitable host cell. A plasmid vector often contains coding DNA and promoter DNA and has one or more restriction sites suitable for inserting foreign DNA. A large number of vectors, including plasmid and fungal vectors, have been described for replication and/or expression in a variety of eukaryotic and prokaryotic hosts. Non-limiting examples include pKK plasmids (Amersham Pharmacia Biotech), pUC plasmids, pET plasmids (Novagen, Inc., Madison, WI), pRSET or pREP plasmids (Invitrogen, San Diego, CA), or pMAL plasmids (New England Biolabs, Beverly, MA), and many appropriate host cells, using methods disclosed or cited herein or otherwise known to those skilled in the relevant art. Recombinant cloning vectors will often include one or more replication systems for cloning or expression, one or more markers for selection in the host, *e.g.* antibiotic resistance, and one or more expression cassettes.

The term "host cell" means any cell of any organism that is selected, modified, transformed, grown, or used or manipulated in any way, for the production of a substance by the cell, for example the expression by the cell of a gene, a DNA or RNA sequence, a protein or an enzyme. Host cells can further be used for screening or other assays, as described *infra*. The host cell may be found *in vitro*, *i.e.*, in tissue culture, or *in vivo*, *i.e.*, in a microbe, plant or animal.

The term "expression system" means a host cell and compatible vector under suitable conditions, *e.g.* for the expression of a protein coded for by foreign DNA carried by the vector and introduced to the host cell. Common expression systems include *E. coli* host cells and plasmid vectors, insect host cells and *Baculovirus* vectors, and mammalian host cells and vectors. In a specific embodiment, the synthebody is expressed in COS-1 or CHO

cells. Other suitable cells include NSO cells, HeLa cells, 293T (human kidney cells), mouse primary myoblasts and NIH 3T3 cells.

The terms "mutant" and "mutation" mean any detectable change in genetic material, *e.g.*, DNA, or any process, mechanism, or result of such a change. This includes gene mutations, in which the structure (*e.g.*, DNA sequence) of a gene is altered, any gene or DNA arising from any mutation process, and any expression product (*e.g.*, protein or enzyme) expressed by a modified gene or DNA sequence. The term "variant" may also be used to indicate a modified or altered gene, DNA sequence, enzyme, cell, etc., *i.e.*, any kind of mutant.

"Sequence-conservative variants" of a polynucleotide sequence are those in which a change of one or more nucleotides in a given codon position results in no alteration in the amino acid encoded at that position.

"Function-conservative variants" are those in which a given amino acid residue in a protein or enzyme has been changed without altering the overall conformation and function of the polypeptide, including, but not limited to, replacement of an amino acid with one having similar properties (such as, for example, polarity, hydrogen bonding potential, acidic, basic, hydrophobic, aromatic, and the like). Amino acids with similar properties are well known in the art. For example, arginine, histidine and lysine are hydrophilic-basic amino acids and may be interchangeable. Similarly, isoleucine, a hydrophobic amino acid, may be replaced with leucine, methionine or valine. Such changes are expected to have little or no effect on the apparent molecular weight or isoelectric point of the protein or polypeptide. Amino acids other than those indicated as conserved may differ in a protein or enzyme so that the percent protein or amino acid sequence similarity between any two proteins of similar function may vary and may be, for example, from 70% to 99% as determined according to an alignment scheme such as by the Cluster Method, wherein similarity is based on the MEGALIGN algorithm. A "function-conservative variant" also includes a polypeptide or enzyme which has at least 60 % amino acid identity as determined by BLAST or FASTA algorithms, preferably at least 75%, more preferably at least 85%, and even more preferably at least 90%, and which has the same or similar properties or functions as the native or parent protein or enzyme to which it is compared.

As used herein, the term "oligonucleotide" refers to a nucleic acid, generally of at least 10, preferably at least 15, and more preferably at least 20 nucleotides, preferably no more than about 100 nucleotides, that is hybridizable to a genomic DNA molecule, a cDNA molecule, or an mRNA molecule having a sequence of interest. Oligonucleotides can

be labeled, *e.g.*, with ^{32}P-nucleotides or nucleotides to which a label, such as biotin, has been covalently conjugated. In one embodiment, a labeled oligonucleotide can be used as a probe to detect the presence of a nucleic acid. In another embodiment, oligonucleotides (one or both of which may be labeled) can be used as PCR primers, either for cloning full length or a fragment of the synthebody, or to detect the presence of nucleic acids encoding the synthebody. In a further embodiment, an oligonucleotide of the invention can form a triple helix with a synthebody-encoding DNA molecule, *e.g.*, for purification purposes. Generally, oligonucleotides are prepared synthetically, preferably on a nucleic acid synthesizer. Accordingly, oligonucleotides can be prepared with non-naturally occurring phosphoester analog bonds, such as thioester bonds, etc.

Constructs

The constructs of the invention can be derived from any type of immunoglobulin molecule, for example, but not limited to, antibodies, T lymphocyte receptors, cell-surface adhesion molecules such as the co-receptors CD4, CD8, CD19, and the invariant domains of MHC molecules. In a preferred embodiment of the invention, the construct is derived from an antibody, which can be any class of antibody, *e.g.*, an IgG, IgE, IgM, IgD or IgA, preferably, the antibody is an IgG. Such antibodies may be in membrane bound (B cell receptor) or secreted form, preferably secreted. Additionally, the antibody may be of any subclass of the particular class of antibodies. In another specific embodiment, the construct is derived from a T lymphocyte receptor.

CDR-grafted variable region genes have been constructed by various methods such as site-directed mutagenesis as described in Jones *et al.*, Nature, 1986, 321:522; Riechmann *et al.*, Nature, 1988, 332:323; *in vitro* assembly of entire CDR-grafted variable regions (Queen *et al.*, Proc. Natl. Acad. Sci. USA, 1989, 86:10029); and the use of PCR to synthesize CDR-grafted genes (Daugherty *et al.*, Nucleic Acids Res., 1991, 19:2471). CDR-grafted antibodies are generated in which the CDRs of the murine monoclonal antibody are grafted onto the framework regions of a human antibody. Following grafting, most antibodies benefit from additional amino acid changes in the framework region to maintain affinity, presumably because framework residues are necessary to maintain CDR conformation, and some framework residues have been demonstrated to be part of the antigen combining site. Such CDR-grafted antibodies have been successfully constructed against various antigens, for example, antibodies against IL-2 receptor as described in Queen *et al.* (Proc. Natl. Acad. Sci. USA, 1989, 86:10029), antibodies against cell surface

receptors-CAMPATH as described in Riechmann *et al.* (Nature, 1988, 332:323); antibodies against hepatitis B in Co *et al.* (Proc. Natl. Acad. Sci. USA, 1991, 88:2869); as well as against viral antigens of the respiratory syncitial virus in Tempest *et al.* (BioTechnology, 1991, 9:267). Thus, in specific embodiments of the invention, the construct comprises a variable domain in which at least one of the framework regions has one or more amino acid residues that differ from the residue at that position in the naturally occurring framework region. The techniques employed in creating CDR-grafted antibodies can be adapted for use in constructs of the invention.

The heterologous amino acid sequence can be inserted into any one or more of the CDR regions of the variable domain variant. It is within the skill in the art to insert the binding site into different CDRs of the variable domain and then screen the resulting modified constructs for the ability to bind to the binding partner of the heterologous amino acid sequence. Thus, one can determine which CDR optimally contains the binding site. In specific embodiments in which the construct is an antibody, a CDR of either the heavy or light chain variable region is modified to contain the amino acid sequence of the binding site. In another specific embodiment, the construct contains a variable domain in which the first, second, or third CDR of the heavy chain variable region or the first, second, or third CDR of the light chain variable region contains the amino acid sequence of the binding site. In another embodiment of the invention, more than one CDR contains the amino acid sequence of the binding site or more than one CDR each contains a different binding site sequence for the same molecule or contains a different binding site sequence for a different molecule. In particular embodiments, two, three, four, five or six CDRs (per heavy chain - light chain pair) are engineered to contain the receptor binding portion of CD28. Corresponding modifications are also contemplated for other immunoglobulin superfamily protein-derived constructs of the invention.

In specific embodiments of the invention, the binding site amino acid sequence is either inserted into the CDR without replacing any of the amino acid sequence of the CDR itself or, alternatively, the binding site amino acid sequence replaces all or a portion of the amino acid sequence of the CDR.

As shown in the Examples, *infra*, in a specific embodiment the heterologous binding sequence is very effective, compared to control, when inserted in CDR3 of the light chain variable region. These results depend on the nature of the variable region framework structure that is depicted in Figure 1B. The control synthetic antibody comprises the original consensus variable region.

Relative efficacy of an CD80/86-binding construct can be evaluated by direct binding assays, such as ELISA, Western blotting, direct binding to cells (that can be detected by radiolabelling or fluorescence labeling) and the like; inhibition assays, *e.g.*, with labeled soluble CD28; and functional assays, including T lymphocyte activation, mixed lymphocyte reactions and *in vivo* models of transplant rejection and autoimmune disease such as rheumatoid arthritis.

After preparing constructs containing modified variable domains, the constructs, can be further altered and screened to select an antibody having higher affinity or specificity. Constructs having higher affinity or specificity for the target binding partner may be generated and selected by any method known in the art. For example, but not by way of limitation, the nucleic acid encoding the construct can be mutagenized, either randomly, *i.e.*, by chemical or site-directed mutagenesis, or by making particular mutations at specific positions in the nucleic acid encoding the construct, and then screening the construct expressed from the mutated nucleic acid molecules for binding affinity for the target molecule. Screening can be accomplished by testing the expressed antibody constructs individually or by screening a library of the mutated sequences, *e.g.*, by phage display techniques (see, *e.g.*, U.S. Patent Nos. 5,223,409; 5,403,484; and 5,571,698; PCT Publication WO 92/01047) or any other phage display technique known in the art.

Accordingly, in a specific embodiment, the construct may have a higher specificity or affinity for its target binding partner than a naturally occurring antibody that specifically binds the same antigen. In another embodiment, the modified antibody exhibits a binding constant for target binding partner ranging from about 1×10^6 to about 1×10^{14} M^{-1}.

The constructs of the invention may also be further modified in any way known in the art, e.g., for the modification of antibodies as long as the further modification does not completely prevent binding of the construct to the particular binding partner. In particular, the constructs of the invention may have one or more amino acid substitutions, deletions, or insertions besides the insertion into or replacement of CDR sequences with the binding sequence. Such amino acid substitutions, deletions, or insertions can be any substitution, deletion, or insertion that does not prevent the specific binding of the construct to the target binding partner. For example, such amino acid substitutions include substitutions of functionally equivalent amino acid residues. One or more amino acid residues can be substituted by another amino acid of a similar polarity that acts as a functional equivalent resulting in a silent alteration. Substitutes for an amino acid may be selected from other members of the class to which the amino acid belongs. For example, the

nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

Additionally, one or more amino acid residues can be substituted by a nonclassical amino acid or chemical amino acid analogs, introduced as a substitution or addition into the immunoglobulin sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, alpha-amino isobutyric acid, 4-aminobutyric acid, 2-aminobutyric acid, 6-amino hexanoic acid, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, beta-alanine, fluoro-amino acids, designer amino acids such as beta-methyl amino acids, C-alpha-methyl amino acids, N-alpha-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

Immunoglobulin Fragment Constructs

As noted above, fragments of an immunoglobulin family protein can be modified to create a construct. For example, such fragments include but are not limited to: $F(ab')_2$ fragments that contain the variable regions of both the heavy and the light chains, the light constant region and the CH1 domain of the heavy chain, which fragments can be generated by pepsin digestion of an antibody; Fab fragments generated by reducing the disulfide bonds of an $F(ab')_2$ fragment (King *et al.*, Biochem. J., 1992, 281:317); and Fv fragments, *i.e.*, fragments that contain the variable region domains of both the heavy and light chains (Reichmann and Winter, J. Mol. Biol., 1988, 203:825; King *et al.*, Biochem, J., 1993, 290:723).

Thus, the present invention includes, but is not limited to, single chain antibodies (SCA) (U.S. Patent 4,946,778; Bird, Science, 1988, 242:423-426; Huston *et al.*, Proc. Natl Acad. Sci. USA, 1988, 85:5879-5883; and Ward *et al.*, Nature, 1989, 334:544-546). Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide. Additionally, the invention also provides heavy chain and light chain dimers and diabodies.

Preferred Immunoglobulin Family Proteins

The immunoglobulin molecule modified to generate the constructs is preferably a monoclonal antibody. The antibody that is modified may be a naturally occurring or previously existing antibody, or may be synthesized from known antibody consensus sequences, such as the consensus sequences for the light and heavy chain variable regions in Figures 1A and 1B, or any other antibody consensus or germline (*i.e.*, unrecombined genomic sequences) sequences (*e.g.*, those antibody consensus and germline sequences described in Kabat *et al.*, 1991, Sequences of Proteins of Immunological Interest, 5th edition, NIH Publication No. 91-3242, pp. 2147-2172).

The invention further provides constructs that are modified chimeric or humanized antibodies. A chimeric antibody is a molecule in which different portions of the antibody molecule are derived from different animal species, such as those having a variable region derived from a murine mAb and a constant region derived from a human immunoglobulin constant region. Techniques have been developed for the production of chimeric antibodies (Morrison *et al.*, Proc. Natl. Acad. Sci. USA, 1984, 81:6851-6855; Neuberger *et al.*, Nature, 1984, 312:604-608; Takeda *et al.*, Nature, 1985, 314:452-454; International Patent Application No. PCT/GB85/00392) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity. In a specific embodiment, the synthebody is a chimeric antibody containing the variable domain of a non-human antibody and the constant domain of a human antibody.

In another embodiment, the construct is derived from a humanized antibody, in which the CDRs of the antibody (except for the one or more CDRs containing the heterologous binding sequence) are derived from an antibody of a non-human animal and the framework regions and constant region are from a human antibody (*see*, U.S. Patent No. 5,225,539).

As noted above, the construct can be derived from a human monoclonal antibody. The creation of completely human monoclonal antibodies is possible through the use of transgenic mice. Transgenic mice in which the mouse immunoglobulin gene loci have been replaced with human immunoglobulin loci provide *in vivo* affinity-maturation machinery for the production of human immunoglobulins.

Immunoglobulin Fusion Protein and Derivative Construct

In certain embodiments, the construct is created by fusing (joining) an immunoglobulin family protein modified to include the heterologous binding sequence to an amino acid sequence of another protein (or portion thereof, preferably an at least 10, 20, or 50 amino acid portion thereof) that is not the modified immunoglobulin, thereby creating a fusion (or chimeric) construct. Preferably, the fusion is via covalent bond (for example, but not by way of limitation, a peptide bond) at either the N-terminus or the C-terminus.

The construct may be further modified, *e.g.*, by the covalent attachment of any type of molecule, as long as such covalent attachment does not prevent or inhibit specific binding of the sythebody to its target antigen. For example, but not by way of limitation, the construct may be further modified, *e.g.*, by glycosylation, acetylation, PEGylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to, specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, etc.

In specific embodiments of the invention, the construct is covalently linked to a therapeutic molecule, for example, to target the therapeutic molecule to a particular cell type or tissue, *e.g.*, an accessory or antigen-presenting cell. The therapeutic molecule can be any type of therapeutic molecule known in the art, for example, but not limited to, a chemotherapeutic agent, a toxin, such as ricin, an antisense oligonucleotide, a radionuclide, an antibiotic, anti-viral, or anti-parasitic, etc.

Structure of the Heterologous Binding (Amino Acid) Sequence

Human CD28 is encoded as a 220 amino acid precursor protein having the typical features of an integral membrane protein (Aruffo and Seed, 1987, Proc. Natl. Acad. Sci. USA, 84:8573-8577) with an N-terminal signal sequence of approximately 27 amino acids. The mature CD28 protein is a 202 amino acid polypeptide chain that consists of a 134 amino acid extracellular domain, a 27 amino acid transmembrane region and a 41 amino acid carboxyl-terminal cytoplasmic tail. The sequence of human CD28 is as follows:

MLRLLLALNL FPSIQVTGNK ILVKQSPMLV AYDNAVNLSC KYSYNLFSRE
FRASLHKGLD SAVEVCVVYG NYSQQLQVYS KTGFNCDGKL GNESVTFYLQ
NLYVNQTDIY FCKIEVMYPP PYLDNEKSNG TIIHVKGKHL CPSPLFPGPS
KPFWVLVVVG GVLACYSLLV TVAFIIFWVR SKRSRLLHSD YMNMTPRRPG

PTRKHYQPYA PPRDFAAYRS (SEQ ID NO: 5)

CD28 binds specifically to CD80 and CD86 receptors through the same binding sequence. Thus CD28/CD80 and CD28/CD86 are protein binding pairs. The CD80 and/or CD86 binding site of CD28 has been identified by mutagenesis and amino acid substitution as the sequence MYPPPY (SEQ ID NO:1), that corresponds to amino acid positions 117-122 and is underlined in the above amino acid sequence of CD28. The sequence was identified by testing the binding of CD28-Ig fusion proteins to CD80 and CD86-Ig fusion portions and to transfected L cells expressing CD80 or CD86. Functional activity was evaluated by measuring IL-2 production by CD28 transfected T lymphocytes in response to CD80 and CD-86-Ig fusion proteins (Kariv *et al.*, J. Immunol., 1996; 157:29-38; Truneh *et al.*, Mol. Immunol., 1996; 33:321-334).

Methods of Producing the Constructs

Constructs can be produced by any method known in the art for the synthesis of immunoglobulins, in particular, by chemical synthesis or by recombinant expression, and are preferably produced by recombinant expression techniques.

Recombinant expression of constructs requires construction of a nucleic acid encoding the construct. Such an isolated nucleic acid that contains a nucleotide sequence encoding the construct can be produced using any method known in the art.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual,* Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook *et al.*, 1989"); *DNA Cloning: A Practical Approach,* Volumes I and II (D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization*, B.D. Hames & S.J. Higgins eds. (1985); *Transcription And Translation,* B.D. Hames & S.J. Higgins, eds. (1984); *Animal Cell Culture,* R.I. Freshney, ed. (1986); *Immobilized Cells And Enzymes,* IRL Press, (1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); F.M. Ausubel *et al.* (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994).

Construct Nucleic Acids

Accordingly, the invention provides nucleic acids that contain a nucleotide sequence encoding a construct of the invention.

A nucleic acid that encodes a construct may be assembled from chemically synthesized oligonucleotides (*e.g.*, as described in Kutmeier *et al.*, BioTechniques, 1994, 17:242), that briefly, involves the synthesis of a set of overlapping oligonucleotides containing portions of the sequence encoding the protein, annealing and ligation of those oligonucleotides, and then amplification of the ligated oligonucleotides by PCR .

Accordingly, the invention provides a method of producing a nucleic acid encoding a construct, the method comprising: (a) synthesizing a set of oligonucleotides, the set comprising oligonucleotides containing a portion of the nucleotide sequence that encodes the construct and oligonucleotides containing a portion of the nucleotide sequence that is complementary to the nucleotide sequence that encodes the construct, and each of the oligonucleotides having overlapping terminal sequences with another oligonucleotide of the set, except for those oligonucleotides containing the nucleotide sequences encoding the N--terminal and C-terminal portions of the synthetic synthebody; (b) allowing the oligonucleotides to hybridize or anneal to each other; and (c) ligating the hybridized oligonucleotides , such that a nucleic acid containing the nucleotide sequence encoding the synthetic synthebody is produced.

Another method for producing a nucleic acid encoding a construct is to modify nucleic acid sequences that encode an immunoglobulin superfamily molecule, e.g., an antibody molecule or at least the variable region thereof, using the "PCR knitting" approach (Figure 2). In "PCR knitting", nucleic acid sequences, such as the consensus variable region sequences shown in Example 1, are used as templates for a series of PCR reactions that result in the selective insertion of a nucleotide sequence that encodes the desired peptide sequence (in this example, the CD80/CD86 binding sequence of CD28) into one or more CDRs of the variable domain. Oligonucleotide primers are designed for these PCR reactions that contain regions complementary to the framework sequences flanking the designated CDR at the 3'ends and sequences that encode the peptide sequence to be inserted at the 5'ends. In addition, these oligonucleotides contain approximately ten bases of complementary sequences at their 5'ends. These oligonucleotide primers can be used with additional flanking primers to insert the desired nucleotide sequence into the selected CDR as shown in Figure 2 resulting in the production of a nucleic acid coding for the synthebody.

Alternatively, a nucleic acid containing a nucleotide sequence encoding a construct can be constructed from a nucleic acid containing a nucleotide sequence encoding, *e.g.,* an antibody molecule, or at least a variable region of an antibody molecule. Nucleic acids containing nucleotide sequences encoding antibody molecules can be obtained either from existing clones of antibody molecules or variable domains or by isolating a nucleic acid encoding an antibody molecule or variable domain from a suitable source, preferably a cDNA library, *e.g.,* an antibody DNA library or a cDNA library prepared from cells or tissue expressing a repertoire of antibody molecules or a synthetic antibody library (see, *e.g.,* Clackson *et al.*, Nature, 1991, 352:624; Hane *et al.*, Proc. Natl. Acad. Sci. USA, 1997, 94:4937), for example, by hybridization using a probe specific for the particular antibody molecule or by PCR amplification using synthetic primers hybridizable to the 3' and 5' ends of the sequence.

If a convenient restriction enzyme site is available in the nucleotide sequence of the CDR, then the sequence can be cleaved with the restriction enzyme and a nucleic acid fragment containing the nucleotide sequence encoding the binding site can be ligated into the restriction site. The nucleic acid fragment containing the binding site can be obtained either from a nucleic acid encoding all or a portion of the protein containing the binding site or can be generated from synthetic oligonucleotides containing the sequence encoding the binding site and its reverse complement.

The nucleic acid encoding the modified antibody optionally contains a nucleotide sequence encoding a leader sequence that directs the secretion of the synthebody molecule.

Construct Expression

Once a nucleic acid encoding a construct is obtained, it may be expressed or it may be introduced into a vector containing the nucleotide sequence encoding the constant region of the antibody (*see, e.g.,* PCT Publications WO 86/05807 and WO 89/01036; and U.S. Patent No. 5,122,464). Vectors containing the complete light or heavy chain for co-expression are available to allow the expression of a complete antibody molecule and are known in the art, for example, pMRRO10.1 and pGammal (*see also*, Bebbington, Methods a companion to Methods in Enzymology, 1991, 2:136-145).

The expression vector can then be transferred to a host cell *in vitro* or *in vivo* by conventional techniques and the transfected cells can be cultured by conventional

techniques to produce a construct of the invention. Specifically, once a variable region of the modified antibody has been generated, the modified antibody can be expressed, for example, by the method exemplified in the Examples (*see also* Bebbington, *supra*). For example, by transient transfection of the expression vector encoding a construct into COS cells, culturing the cells for an appropriate period of time to permit construct expression, and then taking the supernatant from the COS cells, which supernatant contains the secreted, expressed synthebody.

The host cells used to express the recombinant construct of the invention may be either bacterial cells such as *Escherichia coli,* particularly for the expression of recombinant antibody fragments or, preferably, eukaryotic cells, particularly for the expression of recombinant immunoglobulin molecules. In particular, mammalian cells such as Chinese hamster ovary cells (CHO) or COS cells, used in conjunction with a vector in which expression of the construct is under control of the major intermediate early gene promoter element from human cytomegalovirus, is an effective expression system for immunoglobulins (Foecking *et al.*, Gene, 1986, 45:101; Cockett *et al.*, BioTechnology, 1990, 8:662).

A variety of host-expression vector systems may be utilized to express the construct coding sequences of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but may also be used to transform or transfect cells with the appropriate nucleotide coding and control sequences to produce the antibody product of the invention *in situ*. These systems include, but are not limited to, microorganisms such as bacteria *(e.g., E. coli, B. subtilis)* transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing antibody coding sequences; yeast *(e.g., Saccharomyces, Pichia)* transformed with recombinant yeast expression vectors containing antibody coding sequences; insect cell systems infected with recombinant virus expression vectors *(e.g.,* baculovirus) containing the antibody coding sequences; plant cell systems infected with recombinant virus expression vectors *(e.g.,* cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing antibody coding sequences; mammalian cell systems (*e.g.*, COS, CHO, BHK, 293, and 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells *(e.g.,* the metallothionein promoter) or from mammalian viruses *(e.g.,* the adenovirus late promoter; the vaccinia virus 7.5K promoter); and transgenic animal systems, particularly for expression in milk (*e.g.*, U.S. Patent Nos.

5,831,141 and 5,849,992, which describe transgenic production of antibodies in milk; U.S. Patent No. 4,873,316).

Expression of the construct may be controlled by any promoter/enhancer element known in the art, but these regulatory elements must be functional in the host selected for expression. Promoters that may be used to control gene expression include, but are not limited to, cytomegalovirus (CMV) promoter (U.S. Patent Nos. 5,385,839 and 5,168,062), the SV40 early promoter region (Benoist and Chambon, Nature, 1981, 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, *et al.*, Cell, 1980, 22:787-797), the herpes thymidine kinase promoter (Wagner *et al.*, Proc. Natl. Acad. Sci. U.S.A., 1981, 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster *et al.*, Nature, 1982, 296:39-42); prokaryotic expression vectors such as the ß-lactamase promoter (Villa-Komaroff, *et al.*, Proc. Natl. Acad. Sci. U.S.A., 1978, 75:3727-3731), or the *tac* promoter (DeBoer, *et al.*, Proc. Natl. Acad. Sci. U.S.A., 1983, 80:21-25); *see also* "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter; and transcriptional control regions that exhibit hematopoietic tissue specificity, in particular: beta-globin gene control region which is active in myeloid cells (Mogram *et al.*, Nature, 1985, 315:338-340; Kollias *et al.*, 1986, Cell 46:89-94), hematopoietic stem cell differentiation factor promoters, erythropoietin receptor promoter (Maouche *et al.*, Blood, 1991, 15:2557), etc.

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the construct being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions of a construct, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited to, the *E. coli* expression vector pUR278 (Ruther *et al.*, EMBO J., 1983, 2:1791), in which the coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, Nucleic Acids Res., 1985, 13:3101-3109; Van Hleeke & Schuster, J. Biol. Chem., 1989, 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to a matrix of glutathione-agarose beads followed by elution in the presence of free glutathione.

The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The antibody coding sequence may be cloned individually into non-essential regions (for example, the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example, the polyhedrin promoter).

In mammalian host cells, a number of viral-based and non-viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted into the adenovirus genome. Insertion in a non-essential region of the viral genome *(e.g.,* region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the construct in infected hosts *(see, e.g.,* Logan and Shenk, Proc. Natl. Acad. Sci. U.S.A., 1984, 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted antibody coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (*see* Bittner *et al.*, Methods in Enzymol., 1987, 153:516-544).

Additionally, a host cell strain may be chosen that modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications *(e.g.,* glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells that possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines that stably express the antibody may be engineered. Rather than using expression vectors that contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements *(e.g.*, promoter, enhancer sequences, transcription terminators, polyadenylation sites, etc.) and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci, which, in turn, can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines that express the antibody. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that interact directly or indirectly with the antibody.

A number of selection systems may be used, including but not limited to the herpes simplex virus thyrmidine kinase (Wigler *et al.*, Cell, 1977, 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska and Szybalski, Proc. Natl. Acad. Sci. USA, 1962, 48:2026), and adenine phosphoribosyltransferase (Lowy *et al.*, Cell, 1980, 22:817) genes can be employed in tk-, hgprt-, or aprt- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler *et al.*, Proc. Natl. Acad. Sci. USA, 1980, 77:3567; O'Hare *et al.*, Proc. Natl. Acad. Sci. USA, 1981, 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan and Berg, Proc. Natl. Acad. Sci. USA, 1981, 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin *et al.*, J. Mol. Biol., 1981, 150:1); and hygro, which confers resistance to hygromycin (Santerre *et al.*, Gene, 1984, 30:147).

The expression levels of the construct can be increased by vector amplification (for a review, *see* Bebbington and Hentschel, *The Use of Vectors Based on Gene Amplification for the Expression of Cloned Genes in Mammalian Cells in DNA Cloning*, Vol. 3., Academic Press, New York, 1987). When a marker in the vector system expressing a construct is amplifiable, increases in the level of inhibitor present in the culture medium of the host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the construct gene, production of the construct will also increase (Crouse *et al.*, Mol. Cell. Biol., 1983, 3:257).

In a specific embodiment in which the construct is an antibody (immunoglobulin), the host cell may be co-transfected with two expression vectors of the invention, the first vector encoding a heavy chain derived polypeptide and the second vector encoding a light chain derived polypeptide. The two vectors may contain identical selectable markers which enable equal expression of heavy and light chain polypeptides. Alternatively, a single vector may be used that encodes both heavy and light chain polypeptides. In such situations, the light chain should be placed before the heavy chain to avoid an excess of toxic free heavy chain (Proudfoot, Nature, 1986, 322:562; Kohler, Proc. Natl. Acad Sci. USA, 1980, 77:2197). The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

The invention provides a recombinant cell that contains a vector which encodes a synthetic antibody that has a CDR that contains the amino acid sequence of an active binding site from a member of a binding pair.

Viral and Non-Viral Vectors

Preferred vectors, particularly for cellular assays *in vitro* and *in vivo*, are viral vectors, such as lentiviruses, retroviruses, herpes viruses, adenoviruses, adeno-associated viruses, vaccinia virus, baculovirus, and other recombinant viruses with desirable cellular tropism. Thus, a gene encoding a functional or mutant protein or polypeptide domain fragment thereof can be introduced *in vivo*, *ex vivo*, or *in vitro* using a viral vector or through direct introduction of DNA. Expression in targeted tissues can be affected by targeting the transgenic vector to specific cells, such as with a viral vector or a receptor ligand, or by using a tissue-specific promoter, or both. Targeted gene delivery is described in PCT Publication No. WO 95/28494.

Viral vectors commonly used for *in vivo* or *ex vivo* targeting and therapy procedures are DNA-based vectors and retroviral vectors. Methods for constructing and using viral vectors are known in the art (*see, e.g.*, Miller and Rosman, BioTechniques, 1992, 7:980-990). Preferably, the viral vectors are replication-defective, that is, they are unable to replicate autonomously in the target cell. Preferably, the replication defective virus is a minimal virus, *i.e.*, it retains only the sequences of its genome that are necessary for encapsidating the genome to produce viral particles.

DNA viral vectors include an attenuated or defective DNA virus, such as but not limited to, herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus (AAV), and the like. Defective viruses that entirely or

almost entirely lack viral genes are preferred. Defective virus is not infective after introduction into a cell. Use of defective viral vectors allows for administration to cells in a specific, localized area, without concern that the vector can infect other cells. Thus, a specific tissue can be specifically targeted. Examples of particular vectors include, but are not limited to, a defective herpes virus 1 (HSV1) vector (Kaplitt *et al.*, Molec. Cell. Neurosci., 1991, 2:320-330), defective herpes virus vector lacking a glyco-protein L gene, or other defective herpes virus vectors (PCT Publication Nos. WO 94/21807 and WO 92/05263); an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet *et al.* (J. Clin. Invest., 1992, 90:626-630; *see also* La Salle *et al.*, Science, 1993, 259:988-990); and a defective adeno-associated virus vector (Samulski *et al.*, J. Virol., 1987, 61:3096-3101; Samulski *et al.*, J. Virol., 1989, 63:3822-3828; Lebkowski *et al.*, Mol. Cell. Biol., 1988, 8:3988-3996).

Various companies produce viral vectors commercially, including, but not limited to, Avigen, Inc. (Alameda, CA; AAV vectors), Cell Genesys (Foster City, CA; retroviral, adenoviral, AAV, and lentiviral vectors), Clontech (retroviral and baculoviral vectors), Genovo, Inc. (Sharon Hill, PA; adenoviral and AAV vectors), Genvec (France; adenoviral vectors), IntroGene (Leiden, Netherlands; adenoviral vectors), Molecular Medicine (retroviral, adenoviral, AAV, and herpes viral vectors), Norgen (adenoviral vectors), Oxford BioMedica (Oxford, United Kingdom; lentiviral vectors), and Transgene (Strasbourg, France; adenoviral, vaccinia, retroviral, and lentiviral vectors).

Adenovirus vectors. Adenoviruses are eukaryotic DNA viruses that can be modified to efficiently deliver a nucleic acid of the invention to a variety of cell types. Various serotypes of adenovirus exist. Of these serotypes, preference is given, within the scope of the present invention, to using type 2 or type 5 human adenoviruses (Ad 2 or Ad 5) or adenoviruses of animal origin (*see* PCT Publication No. WO 94/26914). Those adenoviruses of animal origin that can be used within the scope of the present invention include adenoviruses of canine, bovine, murine (example: Mav1, Beard *et al.*, Virology, 1990, 75-81), ovine, porcine, avian, and simian (example: SAV) origin. Preferably, the adenovirus of animal origin is a canine adenovirus, more preferably a CAV2 adenovirus (*e.g.*, Manhattan or A26/61 strain, ATCC VR-800, for example). Various replication defective adenovirus and minimum adenovirus vectors have been described (PCT Publication Nos. WO 94/26914, WO 95/02697, WO 94/28938, WO 94/28152, WO 94/12649, WO 95/02697, WO 96/22378). The replication defective recombinant adenoviruses according to the invention can be prepared by any technique known to the person skilled in the art (Levrero *et*

al., Gene, 1991, 101:195; European Publication No. EP 185 573; Graham, EMBO J., 1984, 3:2917; Graham *et al.*, J. Gen. Virol., 1977, 36:59). Recombinant adenoviruses are recovered and purified using standard molecular biological techniques that are well known to one of ordinary skill in the art.

Adeno-associated viruses. The adeno-associated viruses (AAV) are DNA viruses of relatively small size that can integrate, in a stable and site-specific manner, into the genome of the cells that they infect. They are able to infect a wide spectrum of cells without inducing any effects on cellular growth, morphology or differentiation, and they do not appear to be involved in human pathologies. The AAV genome has been cloned, sequenced and characterized. The use of vectors derived from the AAVs for transferring genes *in vitro* and *in vivo* has been described (*see*, PCT Publication Nos. WO 91/18088 and WO 93/09239; U.S. Patent Nos. 4,797,368 and 5,139,941; European Publication No. EP 488 528). The replication defective recombinant AAVs according to the invention can be prepared by cotransfecting a plasmid containing the nucleic acid sequence of interest flanked by two AAV inverted terminal repeat (ITR) regions, and a plasmid carrying the AAV encapsidation genes (rep and cap genes), into a cell line that is infected with a human helper virus (for example an adenovirus). The AAV recombinants that are produced are then purified by standard techniques.

Retrovirus vectors. In another embodiment the gene can be introduced in a retroviral vector, *e.g.*, as described in U.S. Patent No. 5,399,346; Mann *et al.*, Cell, 1983, 33:153; U.S. Patent Nos. 4,650,764 and 4,980,289; Markowitz *et al.*, J. Virol., 1988, 62:1120; U.S. Patent No. 5,124,263; European Publication Nos. EP 453 242 and EP178 220; Bernstein *et al.*, Genet. Eng.,1985, 7:235; McCormick, BioTechnology, 1985, 3:689; PCT Publication No. WO 95/07358; and Kuo *et al.*, Blood, 1993, 82:845. The retroviruses are integrating viruses that infect dividing cells. The retrovirus genome includes two LTRs, an encapsidation sequence and three coding regions (gag, pol and env). In recombinant retroviral vectors, the *gag*, *pol* and *env* genes are generally deleted, in whole or in part, and replaced with a heterologous nucleic acid sequence of interest. These vectors can be constructed from different types of retrovirus, such as, HIV, MoMuLV ("murine Moloney leukemia virus") MSV ("murine Moloney sarcoma virus"), HaSV ("Harvey sarcoma virus"); SNV ("spleen necrosis virus"); RSV ("Rous sarcoma virus") and Friend virus. Suitable packaging cell lines have been described in the prior art, in particular the cell line PA317 (U.S. Patent No. 4,861,719); the PsiCRIP cell line (PCT Publication No. WO 90/02806) and the GP+envAm-12 cell line (PCT Publication No. WO 89/07150). In addition, the

recombinant retroviral vectors can contain modifications within the LTRs for suppressing transcriptional activity as well as extensive encapsidation sequences that may include a part of the gag gene (Bender *et al.*, J. Virol., 1987, 61:1639). Recombinant retroviral vectors are purified by standard techniques known to those having ordinary skill in the art.

Retroviral vectors can be constructed to function as infectious particles or to undergo a single round of transfection. In the former case, the virus is modified to retain all of its genes except for those responsible for oncogenic transformation properties, and to express the heterologous gene. Non-infectious viral vectors are manipulated to destroy the viral packaging signal, but retain the structural genes required to package the co-introduced virus engineered to contain the heterologous gene and the packaging signals. Thus, the viral particles that are produced are not capable of producing additional virus.

Retrovirus vectors can also be introduced by DNA viruses, which permit one cycle of retroviral replication and amplifies transfection efficiency (*see* PCT Publication Nos. WO 95/22617, WO 95/26411, WO 96/39036 and WO 97/19182).

Lentivirus vectors. In another embodiment, lentiviral vectors can be used as agents for the direct delivery and sustained expression of a transgene in several tissue types, including brain, retina, muscle, liver and blood. The vectors can efficiently transduce dividing and nondividing cells in these tissues, and maintain long-term expression of the gene of interest. For a review, *see*, Naldini, Curr. Opin. Biotechnol., 1998, 9:457-63; *see also* Zufferey, *et al.*, J. Virol., 1998, 72:9873-80). Lentiviral packaging cell lines are available and known generally in the art. They facilitate the production of high-titer lentivirus vectors for gene therapy. An example is a tetracycline-inducible VSV-G pseudotyped lentivirus packaging cell line that can generate virus particles at titers greater than 10^6 IU/ml for at least 3 to 4 days (Kafri, *et al.*, J. Virol., 1999, 73: 576-584). The vector produced by the inducible cell line can be concentrated as needed for efficiently transducing non-dividing cells *in vitro* and *in vivo*.

Non-viral vectors. In another embodiment, the vector can be introduced *in vivo* by lipofection, as naked DNA, or with other transfection facilitating agents (peptides, polymers, etc.). Synthetic cationic lipids can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker (Felgner, et. al., Proc. Natl. Acad. Sci. U.S.A., 1987, 84:7413-7417; Felgner and Ringold, Science, 1989, 337:387-388; see Mackey, *et al.*, Proc. Natl. Acad. Sci. U.S.A., 1988, 85:8027-8031; Ulmer *et al.*, Science, 1993, 259:1745-1748). Useful lipid compounds and compositions for transfer of nucleic acids are described in PCT Patent Publication Nos. WO 95/18863 and WO 96/17823, and in U.S. Patent No. 5,459,127.

Lipids may be chemically coupled to other molecules for the purpose of targeting (see Mackey, *et. al., supra*). Targeted peptides, *e.g.*, hormones or neurotransmitters, and proteins such as antibodies, or non-peptide molecules could be coupled to liposomes chemically.

Other molecules are also useful for facilitating transfection of a nucleic acid *in vivo*, such as a cationic oligopeptide (*e.g.*, PCT Patent Publication No. WO 95/21931), peptides derived from DNA binding proteins (*e.g.*, PCT Patent Publication No. WO 96/25508), or a cationic polymer (*e.g.*, PCT Patent Publication No. WO 95/21931).

It is also possible to introduce the vector *in vivo* as a naked DNA plasmid. Naked DNA vectors for gene therapy can be introduced into the desired host cells by methods known in the art, *e.g.*, electroporation, microinjection, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun, or use of a DNA vector transporter (*see, e.g.*, Wu *et al.*, J. Biol. Chem., 1992, 267:963-967; Wu and Wu, J. Biol. Chem., 1988, 263:14621-14624; Canadian Patent Application No. 2,012,311; Williams *et al.*, Proc. Natl. Acad. Sci. USA, 1991, 88:2726-2730). Receptor-mediated DNA delivery approaches can also be used (Curiel *et al.*, Hum. Gene Ther., 1992, 3:147-154; Wu and Wu, J. Biol. Chem., 1987, 262:4429-4432). U.S. Patent Nos. 5,580,859 and 5,589,466 disclose delivery of exogenous DNA sequences, free of transfection facilitating agents, in a mammal. Recently, a relatively low voltage, high efficiency *in vivo* DNA transfer technique, termed electrotransfer, has been described (Mir *et al.*, C.P. Acad. Sci., 1988, 321:893; PCT Publication Nos. WO 99/01157; WO 99/01158; WO 99/01175).

Therapeutic Use of Constructs

The invention also provides methods for treating or preventing diseases and disorders associated with the activity of a particular molecule (CD28) by administration of a therapeutic of the invention. Such therapeutics include the constructs of the invention and nucleic acids encoding the constructs of the invention.

Generally, administration of products of a species origin or species reactivity that is the same species as that of the subject is preferred. Thus, in administration to humans, the therapeutic methods of the invention preferably use a construct that is derived from a human immunoglobulin superfamily protein but may be an immunoglobulin superfamily protein from a heterologous species such as, for example, a mouse, which may or may not be humanized; in other embodiments, the methods of the invention use a modified antibody that is derived from a chimeric or humanized antibody.

Specifically, pharmaceutical compositions containing the constructs of the invention that specifically bind a particular molecule can be used in the treatment or prevention of diseases or disorders associated with the expression of the particular molecule, *e.g.*, binding partner. In particular, in embodiments discussed in more detail in the subsections that follow, synthebodies that specifically bind can be used to treat various conditions, such as autoimmune diseases. Constructs that specifically bind a ligand or receptor can be used to treat or prevent a disease associated with inappropriate activity of the particular ligand or receptor.

The subjects to which the present invention is applicable may be any mammalian or vertebrate speciesthat include, but are not limited to, cows, horses, sheep, pigs, fowl (*e.g.*, chickens), goats, cats, dogs, hamsters, mice, rats, monkeys, rabbits, chimpanzees, and humans. In a preferred embodiment, the subject is a human.

Treatment and Prevention of Undesirable Immune Responses

The invention provides methods of treating various undesirable immune responses and immune dysfunction, particularly autoimmune disease. The method includes administering to a subject in need of such treatment or prevention a therapeutic of the invention, *i.e.*, a construct, that specifically binds to CD80 and/or CD86, which construct comprises a variable domain with a CDR containing the amino acid sequence of a binding site for the binding partner, or a nucleic acid vector encoding such a construct.

Autoimmune diseases, including, but not limited to, rheumatoid arthritis, multiple sclerosis , and variants such as graft versus host interactions or any disease or disorder characterized by deleterious or unwanted T lymphocyte mediated immune responses, can be treated or prevented by administration of the construct of the invention, which construct specifically binds to CD80 and CD86 co-receptors, blocking the interaction between cells expressing CD28 and cells expressing CD80 and/or CD86 and therefore inhibit T lymphocyte activation. Whether a particular therapeutic is effective to treat or prevent a certain type of immunological disorder can be determined by any method known in the art, for example but not limited to, the methods described in *infra*.

In other embodiments of the invention, the subject being treated with the therapeutic may, optionally, be treated with other appropriate immunosuppressive therapeutic agents. Selection of the appropriate therapeutic agents for use in combination therapy may be made by one of ordinary skill in the art, according to conventional pharmaceutical principles. This combination of therapeutic agents may allow the achievement of therapeutic efficacy

with lower dosages of one or both agents, thereby reducing the potential for adverse side effects. Such additional agents include immuno-supressive drugs, such as cyclosporin A and cyclophosphamide; steroids; non-steroidal anti-inflammatory drugs (NSAIDs); and immunosuppressive cytokines such as interleukin-10 (IL-10).

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Gene Therapy

In a specific embodiment, vectors comprising a sequence encoding a construct of the invention are administered to treat or prevent a disease or disorder associated with the expression or function of a molecule to which the construct specifically binds.

10 Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, *see*, Goldspiel *et al.*, Clinical Pharmacy, 1993, 12:488-505; Wu and Wu, Biotherapy, 1991, 3:87-95; Tolstoshev, Ann. Rev. Pharmacol. Toxicol., 1993, 32:573-596; Mulligan, Science, 1993, 260:926-932; 15 and Morgan and Anderson, Ann. Rev. Biochem., 1993, 62:191-217; May, TIBTECH, 1993, 11:155-215. Methods commonly known in the art of recombinant DNA technology that can be used are described in Ausubel *et al.*, (eds.), 1993, Current Protocols in Molecular Biology, John Wiley & Sons, NY; Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY; and in Chapters 12 and 13, Dracopoli *et al.*, (eds.), 1994, 20 Current Protocols in Human Genetics, John Wiley & Sons, NY. Vectors suitable for gene therapy are described above.

In one aspect, the therapeutic vector comprises a nucleic acid that expresses the construct in a suitable host. In particular, such a vector has a promoter operationally linked to the coding sequence for the construct. The promoter can be inducible or 25 constitutive and, optionally, tissue-specific. In another embodiment, a nucleic acid molecule is used in which the antibody coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for expression of the construct from a nucleic acid molecule that has integrated into the genome (Koller and Smithies, Proc. Natl. Acad. Sci. USA, 1989, 86:8932-8935; Zijlstra 30 *et al.*, Nature, 1989, 342:435-438).

Delivery of the vector into a patient may be either direct, in which case the patient is directly exposed to the vector or a delivery complex, or indirect, in which case, cells are first transformed with the vector *in vitro* then transplanted into the patient. These two approaches are known, respectively, as *in vivo* and *ex vivo* gene therapy.

In a specific embodiment, the vector is directly administered *in vivo,* where it enters the cells of the organism and mediates expression of the constructs. This can be accomplished by any of numerous methods known in the art, *e.g.*, by constructing it as part of an appropriate expression vector and administering it so that it becomes intracellular, *e.g.*, by infection using a defective or attenuated retroviral or other viral vector (*see*, U.S. Patent No. 4,980,286), or by direct injection of naked DNA, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont); or coating with lipids or cell-surface receptors or transfecting agents, encapsulation in biopolymers (*e.g.*, poly-β1→4-N-acetylglucosamine polysaccharide; *see*, U.S. Patent No. 5,635,493), encapsulation in liposomes, microparticles, or microcapsules; by administering it in linkage to a peptide or other ligand known to enter the nucleus; or by administering it in linkage to a ligand subject to receptor-mediated endocytosis (*see*, *e.g.*, Wu and Wu, J. Biol. Chem., 1987, 62:4429-4432), etc. In another embodiment, a nucleic acid ligand complex can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (see, *e.g.,* PCT Publication Nos. WO 92/06180, WO 92/22635, WO 92/20316 and WO 93/14188). These methods are in addition to those discussed above in conjunction with "Viral and Non-viral Vectors".

Alternatively, single chain antibody-like constructs can also be administered, for example, by expressing nucleotide sequences encoding single-chain antibodies within the target cell population by utilizing, for example, techniques such as those described in Marasco *et al.* (Proc. Natl. Acad Sci. USA, 1993, 90:7889-7893).

The form and amount of therapeutic nucleic acid envisioned for use depends on the type of disease and the severity of the desired effect, patient state, etc., and can be determined by one skilled in the art.

Formulations and Administration

Therapeutic compositions containing a construct for use in accordance with the present invention can be formulated in any conventional manner using one or more physiologically acceptable carriers or excipients.

Thus, the construct proteins or nucleic acids encoding them and their physiologically acceptable salts and solvents can be formulated for administration by inhalation (pulmonary) or insufflation (either through the mouth or the nose), by transdermal

delivery, or by transmucosal administration, including, but not limited to, oral, buccal, nasal, ophthalmic, vaginal, or rectal administration.

For oral administration, the therapeutics can take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents *(e.g.,* pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers *(e.g.,* lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets can be coated by methods well known in the art. Liquid preparations for oral administration can take the form of, for example, solutions, syrups, emulsions or suspensions, or they can be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations can be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations can also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration can be suitably formulated to give controlled release of the active compound.

For buccal administration the therapeutics can take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the therapeutics according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit can be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin for use in an inhaler or insufflator can be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The therapeutics can be formulated for parenteral administration (*e.g.*, intravenous, intramuscular, subcutaneous, intradermal) by injection, via, for example, bolus injection or continuous infusion. Formulations for injection can be presented in unit dosage form, *e.g.*, in vials or ampoules or in multi-dose containers, with an added preservative. The

compositions can take such forms as excipients, suspensions, solutions or emulsions in oily or aqueous vehicles, and can contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient can be in dry, lyophilized (i.e. freeze dried) powder form for constitution with a suitable vehicle, *e.g.,* sterile pyrogen-free water or saline, before use.

The therapeutics can also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.,* containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the therapeutics can also be formulated as a depot preparation. Such long acting formulations can be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds can be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

In a specific embodiment, the constructs can be delivered in poly-glycolic acid/lactic acid (PGLA) microspheres (*see* U.S. Patent Nos. 5,814,344, 5,100,669, and 4,849,222; PCT Publication Nos. WO 95/11010 and WO 93/07861).

The constructs of the invention may be administered as separate compositions or as a single composition with more than one construct linked by conventional chemical or by molecular biological methods. Additionally, the diagnostic and therapeutic value of the constructs of the invention may be augmented by their use in combination with therapeutic agents used in the treatment of immune dysfunction.

The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc.

Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water-free concentrate in a sealed container such as a vial or sachette indicating the quantity of active agent. Where the composition is administered by injection, a vial of sterile diluent can also be provided so that the ingredients may be mixed prior to administration.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the formulations of the

invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

The compositions may, if desired, be presented in a pack or dispenser device that may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. Composition comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

Many methods may be used to introduce the vaccine formulations of the invention; these include but are not limited to oral, intracerebral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal routes, and via scarification (scratching through the top layers of skin, *e.g.*, using a bifurcated needle) or any other standard routes of immunization.

Effective Dose

The constructs and vectors described herein can be administered to a patient at therapeutically effective doses to treat certain inappropriate immune responses and immune dysfunctions, particularly autoimmune diseases. A therapeutically effective dose refers to that amount of a therapeutic sufficient to result in a healthful benefit in the treated subject.

The precise dose of the constructs to be employed in the formulation depends on the route of administration, and the nature of the patient's disease, and should be decided according to the judgment of the practitioner and each patient's circumstances according to standard clinical techniques. An effective dose is an amount effective to result in inhibition of CD28 binding to CD80/86 *in vivo*; preferably, this dose inhibits T lymphocyte activation. The term "inhibit" or "inhibition" means to reduce by a measurable or observable amount. Thus, inhibition of T lymphocyte activation can mean a reduction in the level of T lymphocyte proliferation, or decreased secretion of IL-2, or both. The ability of a therapeutic composition of the invention to produce this effect can be detected *in vitro*, *e.g.*, using a competitive binding assay with labeled CD28 as exemplified *infra*. Such an assay can be formatted in a solid phase format, in which CD28 or CD80/86, or Ig-chimeric forms thereof, is adsorbed to a solid support, or in a cell-based assay format. Further, experimental evidence

of inhibition includes observing a reduction in inflammation or in symptomatic manifestations of inflammation in an animal model. The degree of inhibition is at least sufficient for measurement; preferably, it is at least about 5%; more preferably from about 5% to about 50%; more preferably still greater than about 50%; and most preferably greater than about 95%. Effective doses may be extrapolated from dose-response curves derived from animal model test systems, including transgenic animal models.

Toxicity and therapeutic efficacy of compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Therapeutics that exhibit large therapeutic indices are preferred. While therapeutics that exhibit toxic side effects can be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration utilized. For any construct used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (*i.e.*, the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma can be measured, for example, by high performance liquid chromatography.

EXAMPLES

The following Examples illustrate the invention without limiting it.

EXAMPLE 1: Construction of the Variable Region Gene Containing the CD28 Receptor-Sequence

The variable region gene encoding a CDR containing the binding domain of CD28 that binds specifically to CD80 and CD86 receptors was constructed with the following cloning steps using standard conditions. First, a "PCR knitting" protocol was developed (see example diagramed in Figure 2) to replace existing CDR sequences with the desired sequences from the CD28 binding site. In this protocol, positive and negative strand oligonucleotide primers were designed that overlap approximately ten residues at the 5' end and contain novel sequences at their 5' ends that encode the peptide sequences to be inserted. At the 3' ends, the oligonucleotides contain sequences homologous to the framework sequences adjacent to the CDR being modified. Two polymerase chain reactions (PCR) were then performed using as primer pairs, one primer that encodes a portion of the peptide sequence and an appropriate primer up- or downstream from the CDR to be modified that corresponds to framework sequences that flank the CDR (Figure 2). The template DNA used for these two PCR reactions was the consensus variable region that has been described previously (Figure 1A and 1B) and was cloned into the shuttle vector pUC19 using standard techniques. PCR reactions were initiated by incubating the reactions for 10 minutes at 95°C and then running 25 cycles of 30 seconds at 95°C, followed by 30 seconds at 55°C and followed by 30 seconds at 72°C. After the 25 cycles, an additional incubation is performed at 72°C for seven minutes. The two PCR reactions produce DNA fragments that overlap by approximately ten base pairs at one of their termini (Figure 2; Table 2) and these fragments were purified from a 2% agarose gel using a QIAquick Gel Extraction kit according to the manufacturer's instructions (Qiagen).

The isolated fragments were then "knitted" together in another PCR reaction in which the flanking primers used in the first two PCR reactions described above were included in the reaction along with the two DNA fragments. PCR reactions were begun by incubating for 10 minutes at 95°C, then 5 cycles were run of 30 seconds at 94°C, followed by 1 minute at 40°C and followed by 30 seconds at 72°C. Twenty-five additional cycles were then performed of 30 seconds at 94°C, followed by 30 seconds at 55°C, and followed by 30 seconds at 72°C. After 25 cycles, an additional incubation is performed for seven minutes at 72°C. The product of this reaction was a longer DNA fragment that results from the joining

of the initial two fragments by selective annealing of the DNA fragments through the overlapping sequences present at one of their termini followed by amplification with the flanking primers (Figure 2).

Table 2. Sequences of primers used for preparation of CD28 constructs. Sequences are shown in 5' to 3' orientation.

Primer (SEQ ID NO:)	Sequence
CD28VLP6 (6)	AAGGAGGAGGATACATAACTTCACAATAATAGGTAGCGAAGTC
CD28VLP7 (7)	CCTCCTCCTTACCTAGACAATTTCGGACAAGGAACCAAGG
VHLP8 (8)	(Reverse -48) AGCGGATAACAATTTCACACAGGA
VLP5 (9)	GGAGTGCCTAGTCGGTTC

To facilitate the cloning of the modified CDR containing the CD28 sequences back into the consensus variable region clone, unique restriction sites were inserted into the flanking sequences, one on either side of each CDR (Figure 2), using the QuikChange™ kit from Stratagene according to the manufacturer's instructions. The "knitted" PCR fragment was then cleaved with the appropriate restriction enzymes (Figure 2) and ligated into the cloned consensus variable region that had been cut with the same restriction enzymes.

The assembled, modified variable region containing the CD28 sequences was then linked to the appropriate constant region clone. For assembly of the light chain of the antibody, a unique *BglII* restriction enzyme site was engineered into the 3' end of the variable region and a *BclI* restriction enzyme site was added to the 5' end of the light chain constant region. An *Eco*RI restriction site and a Kozak sequence were added to the 5'end of the variable region using PCR. When *Bgl*II and *Bcl*I cut their respective cleavage sites, both enzymes leave overhanging ends with the same DNA sequence that allows them to be ligated. The modified light chain variable region was then joined to the light chain constant region (κ chain) by inserting the *Eco*RI/*Bgl*II cut variable region fragment into a vector obtained from Lonza Biologics PLC containing a light chain constant region cut with *Eco*RI and *Bcl*I.

For assembly of the heavy chain of the antibody, a unique *Xho*I restriction enzyme site was engineered into both the 3' end of the heavy chain consensus variable region

and the 5' end of the heavy chain constant region. Similar to the light chain variable region, an *Eco*RI restriction site and Kozak sequence were added to the 5' end of the heavy chain variable region using PCR. The consensus heavy chain variable region clone was digested with *Eco*RI/*Xho*I and the resulting fragment inserted into a second vector, obtained from Lonza Biologics PLC, containing the heavy chain constant region cut with *Eco*RI and *Xho*I.

In a final step, the heavy chain expression vector, containing the heavy chain variable and constant regions, and the light chain expression vector, containing the light chain variable and constant regions, were assembled into a single "double gene" expression vector. To assemble the "double gene" vector, the heavy chain expression vector was cleaved with *Bam*HI and *Not*I. The resulting fragment contains the complete heavy chain expression cassette including the CMV promoter, the assembled heavy chain and a transcriptional terminator. The light chain expression vector was also cleaved with *Bam*HI and *Not*I and after purifying the vector from a small fragment was ligated with the *Bam*HI/*Not*I fragment containing the heavy chain cassette. The resultant "double gene" vector expresses both the heavy and light chains. The modified antibody construct is termed "CD28L3".

A double gene construct was also prepared in an episomal vector, pCEP4 (Invitrogen), for expression of the CD28L3 synthebody. To facilitate the transfer of the CD28L3 double gene construct into pCEP4, pCEP4 was modified by first digesting the vector with *Sal*I and isolating the larger fragment containing the EBV origin of replication and hygromycin selectable marker. A DNA fragment encoding a polylinker with restriction sites for insertion of the CD28L3 double gene construct was ligated to the isolated *Sal*I fragment. The pEE14.1 CD28L3 double gene construct was then digested with *Mlu*I and *Sal*I and the fragment encoding the double gene was ligated with the *Sal*I-*Asc*I digested modified pCEP4. When *Mlu*I and *Asc*I cleave DNA, they leave the same overlapping DNA sequence so they can be ligated together. The resultant vector will contain the same promoters, light and heavy chain genes and terminators as the pEE14.1 construct, but replicates episomally when introduced into eukaryotic cells.

Peptide sequences of variable region containing CD28 binding sequence.

The inserted CD28 sequence in CDR3 of the light chain variable region is indicated by underlining. Peptide sequences of consensus heavy chain (CONVH) and consensus light chain (CONVL) variable regions are shown in Figure 1.

CD28VLCDR3

MetAlaTrpValTrpThrLeuLeuPheLeuMetAlaAlaAlaGlnSerAlaGlnAlaAspIleGlnMet
ThrGlnSerProSerSerLeuSerAlaSerValGlyAspArgValThrIleThrCysArgAlaSerGln
SerIleSerAsnTyrLeuAlaTrpTyrGlnGlnLysProGlyLysAlaProLysLeuLeuIleTyrAla
AlaSerSerLeuGluSerGlyValProSerArgPheSerGlySerGlySerGlyThrArgPheThrLeuThrIleSerSerL
euGlnProGluAspPheAlaThrTyrTyrCysGluValMetTyrProProProTyr
LeuAspAsnPheGlyGlnGlyThrLysValGluIleLys (SEQ ID NO:10)

The nucleotide sequences of the VH and VL consensus sequences are also provided:

CONVH

ATGGCTTGGGTGTGGACCTTGCTATTCCTGATGGCAGCTGCCCAAAGTGCCCA
AGCACAGGTTCAGCTGGTGCAGTCTGGCGCTGAGGTGAAGAAGCCTGGCGCTTCTGT
GAAGGTGTCTTGCAAGGCTTCTGGCTACACATTCACATCTTACGCTATATCTTGGAA
TTGGGTGAGGCAGGCTCCCGGGCAGGGCCTGGAGTGGATGGGCTGGATAAATGGAA
ATGGAGATACAAATTACGCCCAGAAGTTCCAGGGAAGGGTTACTATAACTGCTGAT
ACTTCTACTTCTACTGCTTACATGGAGCTCTCTTCTCTGAGGTCTGAGGATACTGCTG
TTTACTACTGCGCTAGGGCTCCTGGCTACGGCTCTGATTATTGGGGACAGGGAACAC
TGGTTACAGTCTCGAGT (SEQ ID NO:11)

VLCON

ATGGCTTGGGTGTGGACCTTGCTATTCCTGATGGCAGCTGCCCAAAGTGCCCAAGCA
GATATCCAAATGACACAAAGTCCTAGTAGTTTGAGTGCTAGTGTGGGAGATCGGGT
GACAATCACATGTCGGGCTAGTCAAAGTATCAGTAACTATTTGGCTTGGTATCAACA
AAAGCCCGGGAAGGCTCCTAAGTTGTTGATCTATGCTGCTAGTAGTTTGGAGAGTGG
AGTGCCTAGTCGGTTCAGTGGAAGTGGAAGTGGAACACGGTTCACCTTGACCATCA
GTAGTTTGCAACCTGAAGACTTCGCTACCTATTATTGTCAACAATATAACAGTTTGC
CTTGGACCTTCGGACAAGGAACCAAGGTGGAGATCAAG (SEQ ID NO:12)

EXAMPLE 2: Protein Expression

Once constructs were prepared, initial transfections were performed transiently in CHO-K1 cells. Cotransfections were performed using two single gene constructs and a cationic liposomal reagent. Expression was measured at day 3 and day 7 by ELISA assay. The expressed CD28L3 construct was purified using Protein-A or Protein-G column chromatography and characterized by HPLC and Western immunoblotting.

Stable transfectants can be produced in a number of cell lines including but not limited to CHO-K1, NSO and HEK-293. The choice of which cell line to use depends on a number of factors, some of which include: glycosylation patterns, expression level and ability to adapt to serum-free or protein-free media.

An episomal vector was used to produce stable transfectants in 293E cells. Transfections were performed using a cationic liposomal reagent and after transfection cells were grown in the presence of hygromycin B. Three to 4 weeks after transfection, expression was measured by ELISA assay. For increased production, cells were grown in roller bottles and accumulated protein was measured at day 3, 7 and 10 when culture fluid was harvested.

EXAMPLE 3: Direct binding of CD28L3 to $CD80^+CD86^+$ Raji cells

Binding of CD28L3 to Raji B lymphoma cells was determined by flow cytometry. Raji B lymphoma cells were incubated with indicated concentrations of human (A) or murine (B) control antibodies (consensus Ab) and CD28 synthebody The binding of the CD28 synthebody and control Abs to the cells was probed with FITC-labeled goat-anti-human IgG and evaluated by flow cytometry.

Experimental Procedure

Flow cytometry was used to evaluate binding of CD28 synthebody to Raji B lymphoma cells. Raji cells (ATCC) were distributed into 1.5-ml Eppendor tubes at 1 X 10^6 cell each and centrifuged using an Eppendorf microcentrifuge (5415C) at room temperature at 6000 RPM for 1 min. The supernatant was removed by vacuuming and cells were resuspended in 1% BSA-PBS (FACS buffer. BSA, Sigma, Cat. # A-7888; PBS, GIBCO-BRL, Cat.# 14190-144) containing varying concentrations of CD28L3 or human consensus antibodies. Following incubation at 4°C for 40 min., cells were washed once with cold FACS buffer, 0.5 ml/tube by centrifugation at 6000 RPM for 1 min and resuspended in 50 µl of FACS buffer. Two microliters of FITC-labeled goat-anti-human IgG (Southern Biotechnology Associate Inc. Cat# 2043-02) were added to each tube. The cells were

incubated at 4°C for 30 min. and then washed twice with cold FACS buffer. Finally, cells were resuspended in 0.4 ml of FACS buffer, acquired on a flow cytometer (FACScan, Beckton Dickinson) and analyzed using software, Cell Quest (Beckton Dickinson). The results are expressed as mean of percentage of positive cells ± S.D.

Results

In this binding assay, varying concentrations of CD28L3 and human consensus antibody were used to stain $CD80^+CD86^+$ Raji B lymphoma cells. The binding of the two antibodies to the cells was evaluated by flow cytometry. As shown in Figure 3, CD28L3 bound to Raji cells in a dose response manner. The control antibody, human consensus antibody, did not show significant binding to the cells.

EXAMPLE 4: Direct binding of CD28L3 to CD80 and CD86 fusion proteins

Experimental Procedure

In an ELISA plate (Immunlon 4, VWR cat.#), each well was coated with 100 µl of mouse CD80 (R & D Systems, cat. # 740-B1), mouse CD86 (R & D Systems, cat. # 741-B2), or human CD86 (R & D Systems, cat. # 141-B2) fusion proteins in D-PBS (JRH Biosciences, cat. # 59321-78B) at a concentration of 30 nM. Replicate wells without the fusion proteins were included in the assay as an internal negative control. After incubation of the plate at 37°C for 1 hour, the plate was washed once with PBS-0.02% Tween 20 (PBS-T, 150 µl/well) and blocked with 1% BSA-PBS (150 µl/well) at 37°C for 30 min. The plate was then washed with PBS-T. Biotinylated CD28L3 at 300 ng/ml was added to the plate, 100 µl/well. The plate was incubated at 37°C for 2 hours and then washed three times with PBS-T. Following washes, 1:4000 diluted avidin-HRP in D-PBS was added to the plate, 100 µl/well. After incubation at 37°C for 30 min. and three washes with PBS-T, 100 µl of H_2O_2-TMB solution (Pierce cat.# 34021) were added to each well for color development at room temperature for 20 min. The reaction was stopped by

addition of 2 M Sulfuric acid, 35 µl/well. The optical density of each sample was read in duplicate at 450 nm in a microplate reader (Molecular Devices, ThermoMax).

EXAMPLE 5: Blocking of one-way primary mixed lymphocyte reaction by CD28L3 synthebody

A one-way, primary MLR is performed for evaluation of biological efficacy of

CD28L3 synthebody in blocking an allogenic cellular immune response. PBMCs are isolated from individual normal donors by Ficoll gradient centrifugation. Cells from donor A are irradiated at 3000 rads and used as stimulator cells. Cells from donors B and C are used as responder cells. MLR is performed in 96-well tissue culture plates by mixing the stimulator and responder cells at a ratio of 2:1 in the presence of varying concentrations of human IgG1 or CD28L3. Following 5 days incubation, the proliferation of the culture is determined by a fluorescent Alarma Blue assay.

Experimental Procedure

Peripheral blood samples of three normal donors are purchased from Continental Service. In 50-ml conical centrifugation tubes, each blood sample is loaded onto the top of the Ficoll gradient solution (Histopaque-1077, Sigma cat. # 1077-1) at a volume ratio of 1:1. Following centrifugation at 1450 RPM at room temperature for 30 min., white blood cells of each donor are collected from the interface of plasma and the gradient solution, transferred into new 50-ml tubes, and washed three times with 50 mls D-PBS (JRH, cat. # PBS (JRH Biosciences, cat. # 59321-78B) at 1200 RPM for 10 min. The cells are then resuspended in AIM-V medium (GIBCO-BRL cat. # 12055-091) supplemented with 10% of FBS (JRH, cat. # 12106-78P). Cells of donor A are irradiated at 3000 rads in Gammacell at 5 $X10^6$ cells/ml, washed once with the medium and resuspended in AIM-5 medium at 7.5 $X10^6$ cells/ml. The cell density of donors B and C is adjusted to 4 $X10^6$ cells/ml in AIM-5 medium. In a 96-well, U-bottom tissue culture plate, the cells of donor B or C at 2 $X10^5$ cells/well are co-cultured in replicate with irradiated cells of donor A at 4 $X10^5$ cells/well in AIM-5 medium supplemented with 10% of FBS in the presence of varying concentrations of human IgG1 (Sigma cat. # I3889) or CD28L3 synthebody. The cells of individual donors are also cultured in the complete medium containing PHA (Sigma cat.# L2769) at 1 μg/ml. After 5-day culture at 37°C-5% CO_2, 50 μl of culture supernatant are removed from each well and Alamr blue dye (Trek Diagnostic System, cat. # 00-100) added at 50 μl/well. Following 6-hour incubation at 37°C-5% CO_2, the plate is read in a fluorescence plate reader (Molecular Devices, SpectroMax Gemini) with an excitation wavelength of 535 nm and an emission wavelength of 595 nm. The proliferative response of donor B's or C's cells to donor A's cells is calculated by subtracting the response of the donor's cells in the absence of the stimulator cells (irradiated cells) from that of the donor's cells in the presence of the stimulator cells. The results are expressed as mean of fluorescence intensity of triplicate samples ± S.D.

EXAMPLE 6: Evaluation of CD28L3 in PHA and Raji cell-induced IL-2 release assay

CD28L3 synthebody is evaluated for its potential blocking activity on IL-2 release by Jurkat T lymphocytes stimulated with PHA and Raji cells. In a 96-well culture plate, Jurkat T lymphocytes are co-cultured overnight with irradiated Raji cells in the presence of PHA and varying concentrations of human IgG1, mouse CD28-Fc fusion protein, or human CD28L3. The culture supernatants are collected and assayed for levels of IL2 by using a commercial IL-2 ELISA kit.

Experimental Procedure

Jurkat T lymphoma cells (ATCC) are harvested, washed once with complete medium at 1200 RPM for 10 min., and resuspended in complete medium at 1 X 10^6 cells/ml. Raji cells are washed once, resuspended in complete medium at a density of 5 X 10^6 cells/ml, and irradiated at 3000 rads at room temperature. The irradiated Raji cells are washed once and resuspended in the medium at 2 X 10^6 cells/ml. In a 96-well cell culture plate (Costar cat.# 3799), Jurkart cells (5 X 10^4 cells/well) are co-cultured with irradiated Raji cells (1 X 10^4 cells/well) in complete medium containing PHA at 1 μg/ml and varying concentrations of human IgG1 (Sigma cat.# I3889), mouse CD28-Fc fusion protein (R & D systems, cat. # 483-CD) or CD28L3 with a total volume of 200 μl. The Jurkat-Raji culture without PHA served a negative control or background for the assay. Following 18-hour culture at 37°C-5% CO_2, culture supernatants (150 μl/well) are collected for measurement of IL-2 by using an ELISA kit (R & D Systems, cat.# D2050). The optical density at 450 nm of each sample is read by a microplate reader (Molecular Devices, ThermoMax), and the results are expressed as mean of OD 450nm of replicate samples ± S.D.

EXAMPLE 7: Blocking of CD80/CD86-transfectant and anti-CD3 induced proliferation of CD4-positive T lymphocytes by CD28L3

To evaluate biological effect *in vitro*, a CD4 T lymphocyte proliferation assay is performed. In this assay, CD80/CD86-transfected CHO cells are co-cultured with purified human CD4-positive T lymphocytes in the presence of anti-human CD3 mAb. Under the stimulation by anti-CD3 mAb and co-stimulation by CD80/CD86-transfectants, the T lymphocytes proliferate. The CD28L3 synthebody is added to the culture to block CD80/CD86-mediated stimulation of CD4 T lymphocytes.

Experimental Procedure

Peripheral blood mononuclear cells (PBMCs) are isolated from fresh blood samples of normal donors by Ficoll gradient centrifugation as described in the Experimental Procedure for Example 6, *supra*. CD4-positive T lymphocytes are enriched from PBMCs by using a human CD4 T lymphocyte purification system (R & D Systems, cat. # HCD43). The resulting cell preparation is evaluated by flow cytometry for CD3 and CD4 expression. The $CD4^+$ T lymphocyte preparations with a purity of greater than 85% are used in the assay. In a 96-well culture plate coated with anti-CD3 mAb (Ortho Biotechnologies), the purified $CD4^+$ T lymphocytes (5×10^4 cells/well) are co-cultured with CD80/CD86-transfectants (CD80/CD86-CHO, 1×10^5 cells/well) in AIM-V medium (GIBCO-BRL cat. # 12055-091) supplemented with 10% FBS in the presence of varying concentrations of human IgG1 or human CD28L3 synthebody. The cultures without anti-CD3 or CD80/CD86-CHO are used as negative controls, and PBMCs stimulated with free anti-CD3 serves a positive control in the assay. Following 66-hour culture at 37°C-5% CO_2, the cell cultures are pulsed with ^3H-thymidine at 0.5 μCi/well and incubated at 37°C-5% CO_2 for additional 6 hours. The cells are harvested onto glass fiber filter papers (Wallac, cat.# 1450-421), washed and dried. The radioactivity of each sample in replicate is determined by a beta scintillation counter (Microbeta, Wallac Inc.). The results are expressed as mean of replicate samples ± S.D.

EXAMPLE 8: Evaluation of biological activities of CD28L3 synthebody in animal models of graft versus host disease (GVHD) and rheumatoid arthritis (RA)

Two animal models are selected to evaluate potential therapeutic efficacy of human CD28L3 synthebody in treatment of GVHD and RA.

Experimental Procedure

GVHD model. A murine model of *Parent-into-F1* GVHD is used for the evaluation of potential therapeutic efficacy of CD28L3 synthebody to ameliorate acute GVHD. To induce acute GVHD, 7×10^7 splenic mononuclear cells (SMNCs) from C57BL/6 mice are injected intravenously on day 0 into a group of BDF1 mice (n = 20) that have been irradiated at a dose of 4 Gy. The irradiated recipient mice receive an intravenous injection of a murine CD28L3 construct (n = 10) or mouse IgG1 (n = 10) at 100 μg each one day before SMNCs injection (day –1) and every 3 days for 6 additional doses. Mouse survival and body weight are monitored daily. The results are expressed as survival rates over a period of 6 - 8 weeks of observation time.

RA model. A murine model of collagen-induced arthritis (CIA) is used for

the evaluation of potential therapeutic efficacy of CD28L3 synthebody in treatment of rheumatoid arthritis. To induce arthritis, a group of DBA/1 Lac J mice (n = 20, The Jackson Laboratories) are injected intradermally with native type II collagen (Sigma, 100 μg per animal) emulsified with complete Freund's adjuvant on day 0. The mice are then boosted intraperitoneally with native type II collagen (50 μg/mouse) on day 21. Another group of mice are given PBS and serve as negative control for induction of CIA. The collagen-injected mice receive an intravenous injection of murine CD28L3 (100 μg/mouse, n = 10) or mouse IgG1 (100 μg/mouse, n = 10) on day –1 and every 3 days for 8 additional doses. Over a period of 6-8 weeks, the mice are observed two or three times each week for presence of distal joint swelling and erythema. Swelling is quantitated by measuring foot thickness and ankle width with a constant tension caliper (Dyer, Lancaster, PA). The severity of arthritis in the mice is assessed according to the criteria described in Section 15.5.11 in Current Protocols in Immunology.

EXAMPLE 9: Human Clinical Trial

Renal transplant may be characterized by acute or chronic rejection. Acute rejection is associated with preexisting antibodies. Chronic rejection depends upon activation of T lymphocytes that recognize the transplant as foreign. Disruption of T lymphocyte activation by a CD28 construct is expected to block activation of T lymphocytes. In the phase II clinical trial, patients receive a dose of a CD28 construct (10-400mg) intravenously one day prior to transplant, and chronically every two to four weeks following transplantation. Patients are randomized to usual therapy or usual therapy plus CD28 construct. There are 100 patients in each group. Patients are monitored for indices of rejection (T lymphocyte activation in response to lectin, T lymphocyte presence within punch biopsies from the grafts and renal function studies). Results of the two groups are compared using analysis of variance methods.

* * *

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

All patents, applications, publications, test methods, literature, and other materials cited herein are hereby incorporated by reference.

WHAT IS CLAIMED IS:

1. A variant of an immunoglobulin variable domain, said immunoglobulin variable domain comprising (A) at least one CDR region and (B) framework regions flanking said CDR, said variant comprising:

(a) said CDR region having added or substituted therein at least one binding sequence and

(b) said flanking framework regions, wherein said binding sequence is heterologous to said CDR and is a binding sequence from a binding site of a binding pair, and wherein said binding sequence is a CD80 and/or CD86 receptor-binding portion of CD28.

2. A variant as defined in claim 1, wherein (i) one or more amino acid residues in one or more of said flanking framework regions has been substituted or deleted, (ii) one or more amino acid residues has been added in one or more of said flanking framework regions, or (iii) a combination of (i) and (ii).

3. A variant as defined in claim 1, wherein (i) one or more amino acid residues in one or more framework regions other than said framework regions flanking said CDR has been substituted or deleted, (ii) one or more amino acid residues has been added in one or more framework regions other than said framework regions flanking said CDR, or (iii) a combination of (i) and (ii).

4. A variant as defined in claim 1, wherein (i) one or more amino acid residues in one or more of said flanking framework regions has been substituted or deleted, (ii) one or more amino acid residues has been added in one or more of said flanking framework regions, or (iii) a combination of (i) and (ii); and wherein (iv) one or more amino acid residues in one or more framework regions other than said framework regions flanking said CDR has been substituted or deleted, (v) one or more amino acid residues has been added in one or more framework regions other than said framework regions flanking said CDR, or (vi) a combination of (iv) and (v).

5. A variant of an immunoglobulin variable domain, said immunoglobulin variable domain comprising (A) at least one CDR region and (B) framework regions flanking said CDR, said variant comprising:

(a) said CDR region having added or substituted therein at least one amino acid sequence which is heterologous to said CDR and

(b) said flanking framework regions,

wherein said heterologous sequence is capable of binding to a target sequence or molecule, and

wherein said heterologous sequence is a CD80 and/or CD86 receptor-binding portion of CD28.

6. A variant as defined in claim 5, wherein (i) one or more amino acid residues in one or more of said flanking framework regions has been substituted or deleted, (ii) one or more amino acid residues has been added in on or more of said flanking framework regions, or (iii) a combination of (i) and (ii).

7. A variant as defined in claim 5, wherein (i) one or more amino acid residues in one or more framework regions other than said framework regions flanking said CDR has been substituted or deleted, (ii) one or more amino acid residues has been added in one or more framework regions other than said framework regions flanking said CDR, or (iii) a combiantion of (i) and (ii).

8. A variant as defined in claim 5, wherein (i) one or more amino acid residues in one or more of said flanking framework regions has been substituted or deleted, (ii) one or more amino acid residues has been added in one or more of said flanking framework regions, (iii) a combination of (i) and (ii); and wherein (iv) one or more amino acid residues in one or more framework regions other than said framework regions flanking said CDR has been substituted or deleted, (v) one or more amino acid residues has been added in one or more framework regions other than said framework regions flanking said CDR, or (vi) a combination of (iv) and (v).

9. A variant as defined in claim 5, wherein said receptor-binding portion of CD28 has a sequence corresponding to about amino acid residue 117 to about amino acid residue 122 of CD28.

10 A variant as defined in claim 5, wherein said receptor binding portion of CD28 has an amino acid sequence EVMYPPPYLDN (SEQ ID NO:2).

11. A variant as defined in claim 5, wherein said receptor-binding portion of angiogenin is in more than one CDR.

12. A variant as defined in claim 5, wherein said receptor-binding portion of angiogenin is in a CDR of a light chain variable region.

13. A variant as defined in claim 5, wherein said heterologous sequence comprises the amino acid sequence EVMYPPPYLDN (SEQ ID NO:2) in CDR3 of a human light chain variable region.

14. A variant as defined in claim 5, wherein said heterologous sequence is capable of specifically binding to said target sequence or molecule.

15. A variant as defined in claim 5, wherein said CDR region is CDR 1.

16. A variant as defined in claim 5, wherein said CDR region is CDR 2.

17. A variant as defined in claim 5, wherein said CDR region is CDR 3.

18. A variant as defined in claim 5, which is an antibody.

19. A molecule comprising a variant as defined in claim 5.

20. A molecule comprising a variant as defined in claim 6.

21. A molecule comprising a variant as defined in claim 7.

22. A molecule comprising a variant as defined in claim 8.

23. A molecule comprising a variant as defined in claim 9.

24. A molecule comprising a variant as defined in claim 10.

25. A molecule comprising a variant as defined in claim 13.

26. A molecule as defined in claim 19, further comprising one or more constant domains from an immunoglobulin.

27. A molecule as defined in claim 19, further comprising a second variable domain linked to said variant.

28. A molecule as defined in claim 19, further comprising a second variable domain linked to said variant, and one or more constant domains from an immunoglobulin.

29. A molecule as defined in claim 19, wherein said heterologous sequence is capable of specifically binding to said target sequence or molecule.

30. A molecule as defined in claim 19, wherein said CDR region is CDR 1.

31. A molecule as defined in claim 19, wherein said CDR region is CDR 2.

32. A molecule as defined in claim 19, wherein said CDR region is CDR 3.

33. A molecule as defined in claim 19, which is an antibody.

34. A molecule as defined in claim 19, which comprises an amino acid sequence as depicted in SEQ ID NO:10.

35. A molecule as defined in claim 19, which is derived from a human antibody.

36. A molecule as defined in claim 19, which is derived from a chimeric or a humanized antibody.

37. An immunoglobulin comprising a heavy chain and a light chain, wherein said heavy chain comprises a variant as defined in claim 5 and three constant domains from an immunoglobulin heavy chain, and said light chain comprises a second variable domain associated with said variant and a constant domain from an immunoglobulin light chain.

38. An immunoglobulin comprising a heavy chain and a light chain, wherein said light chain comprises a variant as defined in claim 5 and a constant domain from an immunoglobulin light chain, and said heavy chain comprises a second variable domain associated with said variant and three constant domains from an immunoglobulin heavy chain.

39. An isolated nucleic acid encoding a variant as defined in claim 1.

40. An isolated nucleic acid encoding a variant as defined in claim 5.

41. An isolated nucleic acid encoding a molecule as defined in claim 19.

42. An isolated nucleic acid encoding an immunoglobulin as defined in claim 37.

43. An isolated nucleic acid encoding an immunoglobulin as defined in claim 38.

44. A cell containing nucleic acid as defined in claim 39.

45. A cell containing nucleic acid as defined in claim 40.

46. A cell containing nucleic acid as defined in claim 41.

47. A cell containing nucleic acid as defined in claim 42.

48. A cell containing nucleic acid as defined in claim 43.

49. A recombinant non-human host containing nucleic acid as defined in claim 39.

50. A recombinant non-human host containing nucleic acid as defined in claim 40.

51. A recombinant non-human host containing nucleic acid as defined in claim 41.

52. A recombinant non-human host containing nucleic acid as defined in claim 42.

53. A recombinant non-human host containing nucleic acid as defined in claim 43.

54. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of a variant as defined in claim 1, and a pharmaceutically acceptable carrier.

55. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of a variant as defined in claim 5, and a pharmaceutically acceptable carrier.

56. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of a variant as defined in claim 9, and a pharmaceutically acceptable carrier.

57. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of a variant as defined in claim 10, and a pharmaceutically acceptable carrier.

58. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of a variant as defined in claim 13, and a pharmaceutically acceptable carrier.

59. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of a molecule as defined in claim 19, and a pharmaceutically acceptable carrier.

60. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of an immunoglobulin as defined in claim 37, and a pharmaceutically acceptable carrier.

61. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of an immunoglobulin as defined in claim 38, and a pharmaceutically acceptable carrier.

62. A method of treating or preventing a disease in a subject in need of such treatment or prevention, said method comprising administering to said subject a disease treating or preventing effective amount of a variant as defined in claim 1, wherein (i) said disease is caused directly or indirectly by an agent, (ii) a symptom of said disease is caused by an agent, or (iii) said disease produces a physical, chemical, or biological response, wherein said agents or response include said target sequence or molecule.

63. A method of treating or preventing a disease in a subject in need of such treatment or prevention, said method comprising administering to said subject a disease treating or preventing effective amount of a variant as defined in claim 5, wherein (i) said disease is caused directly or indirectly by an agent, (ii) a symptom of said disease is caused by an agent, or

(iii) said disease produces a physical, chemical, or biological response, wherein said agents or response include said target sequence or molecule.

64. A method of treating or preventing a disease in a subject in need of such treatment or prevention, said method comprising administering to said subject a disease treating or preventing effective amount of a molecule as defined in claim 19, wherein (i) said disease is caused directly or indirectly by an agent, (ii) a symptom of said disease is caused by an agent, or (iii) said disease produces a physical, chemical, or biological response, herein said agents or response include said target sequence or molecule.

65. A method of treating or preventing a disease in a subject in need of such treatment or prevention, said method comprising administering to said subject a disease treating or preventing effective amount of an immunoglobulin as defined in claim 37, wherein (i) said disease is caused directly or indirectly by an agent, (ii) a symptom of said disease is caused by an agent, or (iii) said disease produces a physical, chemical, or biological response, wherein said agents or response includes said target sequence or molecule.

66. A method of treating or preventing a disease in a subject in need of such treatment or prevention, said method comprising administering to said subject a disease treating or preventing effective amount of an immunoglobulin as defined in claim 38, wherein (i) said disease is caused directly or indirectly by an agent, (ii) a symptom of said disease is caused by an agent, or (iii) said disease produces a physical, chemical, or biological response, wherein said agents or response include said target sequence or molecule.

67. A method of treating or preventing a disease in a subject in need of such treatment or prevention, said method comprising administering to said subject a disease treating or preventing effective amount of a nucleic acid as defined in claim 39, wherein (i) said disease is caused directly or indirectly by an agent, (ii) a symptom of said disease is caused by an agent, or (iii) said disease produces a physical, chemical, or biological response, wherein said agents or response include said target sequence or molecule.

68. A method of treating or preventing a disease in a subject in need of such treatment or prevention, said method comprising administering to said subject a disease treating or preventing effective amount of a pharmaceutical composition as defined in claim 54, wherein (i) said disease is caused directly or indirectly by an agent, (ii) a symptom of said disease is caused by an agent, or (iii) said disease produces a physical, chemical, or biological response, wherein said agents or response include said target sequence or molecule.

69. A method of treating or preventing a disease in a subject in need of such treatment or prevention, said method comprising administering to said subject a disease treating or preventing effective amount of a vaccine as defined in claim 59, wherein (i) said disease is caused directly or indirectly by an agent, (ii) a symptom of said disease is caused by an agent, or (iii) said disease produces a physical, chemical, or biological response, wherein said agents or response include said target sequence or molecule.

FIGURE 1. Amino acid sequences of consensus heavy chain (CON VH) and consensus light chain (CON VL) variable regions. CDR sequences are underline, in boldface font.

A. CONVH (SEQ ID NO:1)

MetAlaTrpValTrpThrLeuLeuPheLeuMetAlaAlaAlaGlnSerAlaGlnAlaGlnValGlnLeuValGlnSerGlyAlaGluValLysLysProGlyAlaSerValLysV
alSerCysLysAlaSerGlyTyrThrPhe Thr**SerTyrAlaIleSerTrpAsn**TrpValArgGlnAlaProGlyGlnGlyLeuGluTrpMetGly**TrpIle**
AsnGlyAsnGlyAspThrAsnTyrAlaGlnLysPheGlnGlyArgValThrIleThrAlaAspThrSer
ThrSerThrAlaTyrMetGluLeuSerSerLeuArgSerGluAspThrAlaValTyrTyrCysAlaArg**Ala**
ProGlyTyrGlySerAspTyrTrpGlyGlnGlyThrLeuValThrValSerSer

B. CONVL (SEQ ID NO:2)

MetAlaTrpValTrpThrLeuLeuPheLeuMetAlaAlaAlaGlnSerAlaGlnAlaAspIleGlnMetThrGlnSerProSerSerLeuSerAlaSerValGlyAspAr
gValThrIleThrCys**ArgAlaSerGlnSerIleSer AsnTyrLeuAla**TrpTyrGlnGlnLysProGlyLysAlaProLysLeuLeuIleTyr**AlaAlaSerSerLeu**
GluSerGlyValProSerArgPheSerGlySerGlySerGlyThrArgPheThrLeuThrIleSerSerLeuGlnProGluAspPheAlaThrTyrTyrCys**GlnGlnTy**
rAsnSerLeuProTrpThrPheGlyGlnGlyThr LysValGluIleLys

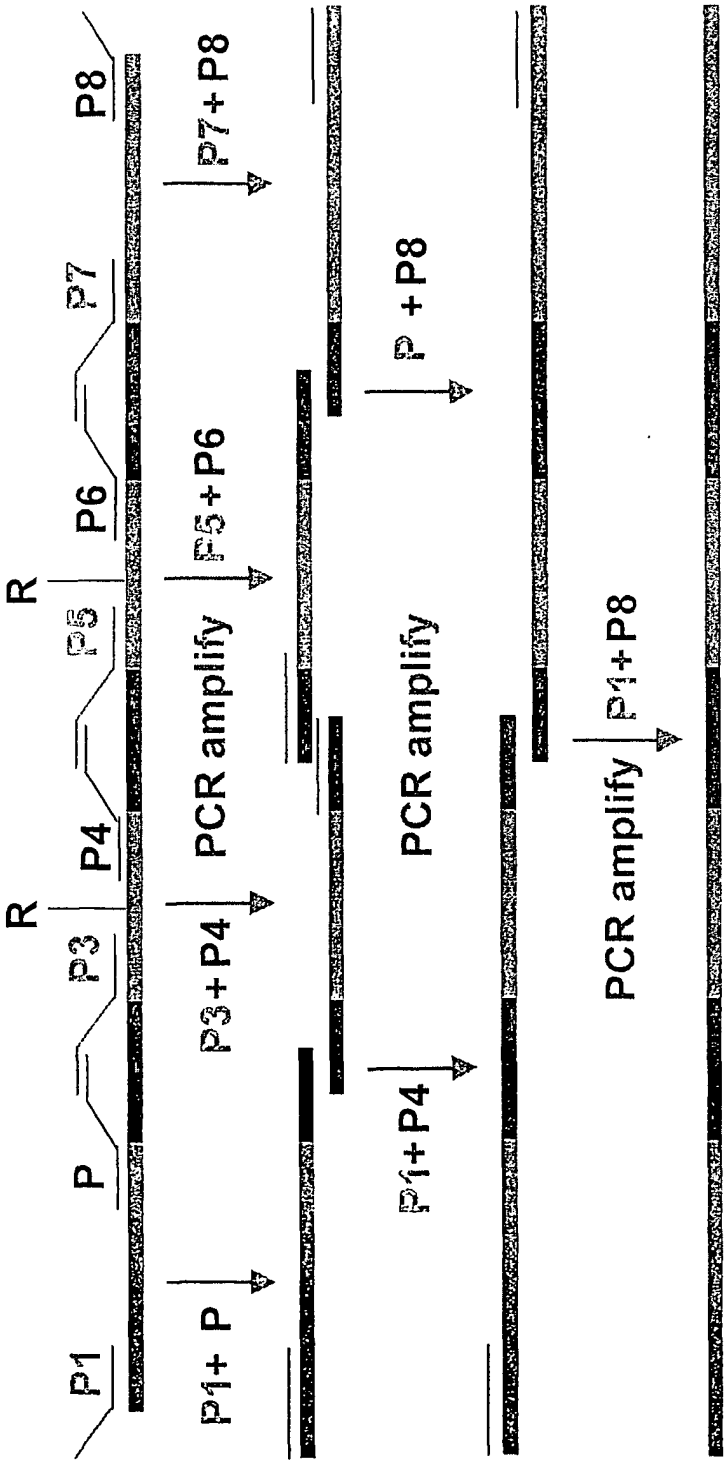
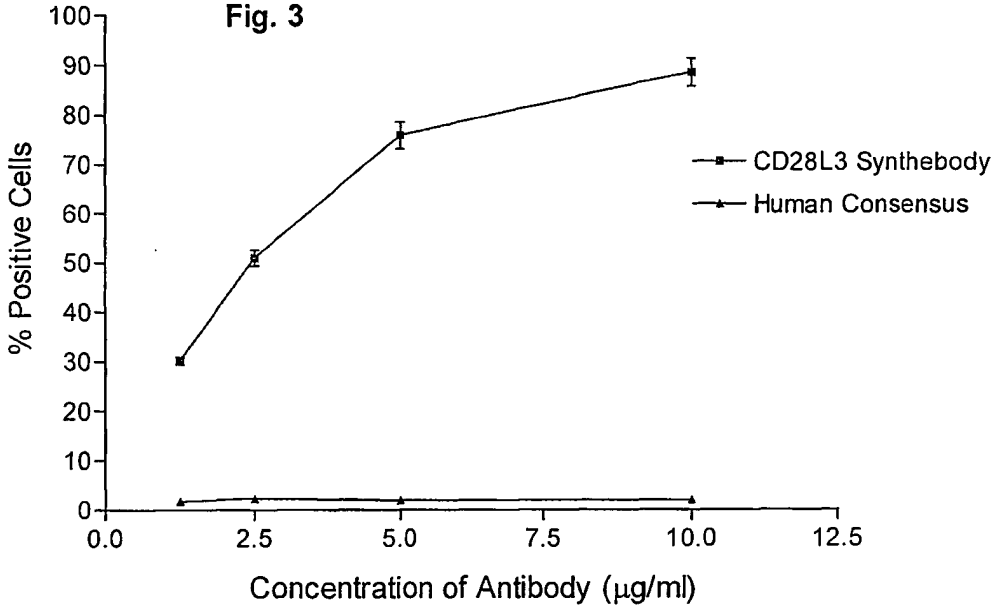

P1
P
R
P3
P4
P5
R
P6
P7
P8
P1+ P
P3+P4
PCR amplify
P5+P6
P7+P8
P1+P4
PCR amplify
P + P8
PCR amplify
P1+P8

Fig. 3


100
90
80
70
60
50
40
30
20
10
0
% Positive Cells
0.0
2.5
5.0
7.5
10.0
12.5
Concentration of Antibody (μg/ml)
CD28L3 Synthebody
Human Consensus